DIFFERENTIAL EXPRESSION OF PUTATIVE CLOTTING PROTEIN GENE IN *VIBRIO PARAHAEMOLYTICUS* CHALLENGED GIANT FRESH-WATER PRAWN, *MACROBRACHIUM ROSENBERGII*

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ABSTRACT

The growth of the freshwater prawn industry has not reached its great heights compared to the marine shrimps because of agriculturists' innate fear of disease that will hamper the growth of the industry. High concentrations of infectious microorganisms (yeast, viruses and bacteria) in crustaceans during hot and cool seasons have resulted in a huge decline in prawn farming. Vibriosis, early mortality syndrome (EMS) and gastroenteritis due to zoonotic outbreaks have been a major threat to the rapid expanding production of freshwater prawns. The innate immune defense which leads to a lot of possible solutions needs to be thoroughly investigated for in-depth understanding of the defense mechanism of these prawns. This thesis investigates one of them namely the coagulation cascade which is a key mechanism in the invertebrate immune defense for avoiding loss of hemolymph particularly the clotting protein which activates a concatenate enzymatic reaction with the proPO system which leads to the production of the black-brownish pigments called melanin. In the current study, the putative clotting protein gene (CP gene) was sequenced from the fresh-water prawn transcriptome unigenes using Illumina's Solexa sequencing technology. The putative clotting protein gene consisted of 5609 base pair with an open reading frame (ORF) length of 5139 bp. Putative CP encoded polypeptide has an estimated molecular mass of 189.9 k Da and 1712 amino acids with a predicted isoelectric point of 5.44. The deduced amino acid sequences of the clotting protein gene were aligned with other CP family members indicating the highest sequence similarity with A7YIH6 (*Penaeus japonicus* clottable protein) , B5KMA2 (*Penaeus monodon* clottable protein) , Q9U572 (*Penaeus monodon* hemolymph clottable protein), A8DR94 (*Litopenaeus vannamei* hemolymph clottable protein) and Q9UAR3 (*Pacifastacus leniusculus* clotting protein). DNAstar protein analysis showed that *M. rosenbergii* CP is a protein with a hydrophilic structure and

good antigenicity index. Analysis using PROSITE to identify the domains on the CP protein showed that positions 81 to 782 corresponds to the vitellogenin domain and positions from 1439 to 1635 correspond to the VWFD domain. Neibor –Joining phylogenetic tree of Clotting protein based on the vitellogenin domain of organism group ranging from invertebrate to vertebrate constructed on the basis of Poisson model and built at a boot strap of 1000. The tree represents tr_Q9UAR3 (*Pacifastacus leniusculus* clotting protein) as the closest ancestral protein.The results of the relative real-time PCR analysis clearly showed that CP expression occurs predominantly in most tissues of *Macrobrachium rosenbergii* but with remarkably highest expression in stomach followed by in pleopods. According to the outcome of the gene expression analysis, the mRNA transcription of the clotting protein gene in *Vibrio parahaemolyticus* challenged *Macrobrachium rosenbergii* was significantly induced in the stomach. The overall results of the current study indicate that the CP is without doubt an essential immune gene involved in the immune response against *V. parahaemolyticus* infection in *Macrobrachium rosenbergii.*

ABSTRAK

Pertumbuhan industri udang air tawar tidak mencapai tahap yang lebih tinggi berbanding dengan udang laut kerana agrikuturis yang mempunyai ketakutan semula jadi penyakit yang akan menghalang pertumbuhan industri ini. Semua sedia maklum, krustasia terdiri daripada kepekatan tinggi mikroorganisma berjangkit (yis, virus dan bakteria) semasa musim panas dan sejuk menyebabkan kemerosotan besar dalam ternakan udang. Penyakit disebabkan Vibriosis, sindrom kematian awal (EMS) dan gastroenteritis disebabkan wabak zoonotik telah menjadi ancaman utama kepada pengeluaran berkembang pesat udang air tawar. Pertahanan imun semula jadi yang membawa kepada banyak penyelesaian, mungkin perlu disiasat dengan teliti untuk pemahaman yang mendalam mekanisme pertahanan udang ini. Tesis ini menyiasat salah satu mekanisme tersebut iaitu pembekuan kaskade yang merupakan mekanisme utama dalam pertahanan imun invertebrat yang bertujuan untuk mengelakkan kehilangan hemolymph. Proses ini juga mengaktifkan reaksi enzim bergabung - sistem PROPO - yang membawa kepada pengeluaran pigmen hitam keperangan dipanggil melanin.

Dalam kajian terkini, gen putative protein pembekuan (CP gen) telah dijujuk dari transkriptom unigene udang air tawar menggunakan teknologi penjujukan Solexa Illumina. Gen putataif CP terdiri daripada 5609bp dengan rangka bacaan terbuka (ORF) panjang 5139 bp. Polipeptida Putative CP mengkodkan polipeptida dengan anggaran jisim molekul sebanyak 189.9 kDa dan 1712 asid amino dengan ramalam titik isoelectric 5.44. Jujukan asid amino gen protein clotting yang dijajarkan dengan ahli keluarga CP lain menunjukkan urutan persamaan yang tertinggi di A7YIH6 (Protein pembekuan *Penaeus japonicus*), B5KMA2 (Protein pembekuan *Penaeus monodon*),

Q9U572 (Protein pembekuan haemolymph *Penaeus monodon*), A8DR94 (Protein pembekuan haemolymph *Litopenaeus vannamei*) dan Q9UAR3 (Protein pembekuan *Pacifastacus leniusculus*). DNAstar analisis protein menunjukkan bahawa CP *M. rosenbergii* adalah protein dengan struktur hidrofilik dan mempunyai indeks antigenicity yang baik. Analisis menggunakan PROSITE untuk mengenal pasti domain pada protein CP itu menunjukkan bahawa kedudukan 81-782 sepadan dengan domain vitellogenin dan kedudukan 1439-1635 sesuai dengan domain VWFD. Pokok filogenetik Neighbour joining bagi protein Clottting berdasarkan domain vitellogenin yang meliputi kumpulan organisma invertebrate kepada vertebrate berasaskan model Poisson dibina menggunakan tali pengikat 1000. Pokok ini diwakili tr_Q9UAR3 (Pacifastacus leniusculus pembekuan protein) sebagai protein keturuna yang paling dekat. Keputusan analisis *Macrobrachium rosenbergii* dengan ekspresi tertinggi di perut diikuti dengan ekspresi di pleopod. Berdasarkan hasil analisis ekspresi gen, transkripsi mRNAgen protein pembekuan dalam *Macrobrachium rosenbergii* yang dijangkiti dengan *Vibrio parahaemolyticus* terhasil dengan banyak secara signifikan dalam perut. Keputusan keseluruhan kajian menunjukkan bahawa CP adalah gen imun penting yang terlibat dalam tindak balas imun terhadap jangkitan *V. parahaemolyticus* dalam *Macrobrachium rosenbergii*.

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α	Alpha
ºC	Degree centigrade
µl	Microliter (10^{-6} l)
10X	Ten times
5X	Five times
A	Adenine
A260	Number of absorption in 260 nm
A_{260}/A_{280}	A_{260}/ number of absorption in 280 nm
aa	Amino acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cds	Coding sequence
CP	Clotting protein
dH2O	Distilled water
DNA	Deoxy-ribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
E	PCR efficiency
FAO	Food and agriculture organization
F	Forward
g	Gram
G	Guanine / guanosine
h	Hour
kDa	Kilo Dalton
l	Liter
M	Molar

mg	Milligram (10^{-3} g)
mM	Milimolar (10^{-6} M)
min	Minute
mg	Milligram (10^{-3} g)
ml	Milliliter (10^{-3} l)
nm	Nanometer (10^{-9} m)
mRNA	Messenger ribonucleic acid
M. rosenbergii	*Macrobrachium rosenbergii*
NCBI	National Center for Biotechnology Information
ng	Nanogram (10^{-9} M)
nm	Nanometer (10^{-9} m)
NTC	No-template control
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	*Puissance hydrogen*
pI	Isoelectric point
Pri	Primer
qPCR	Quantitative polymerase chain raction
R	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature / reverse transcriptase
RT-PCR	Reverse transcriptase PCR
s	Second
S.D.	Standard deviation
SDS	Software-defined storage
T	Thymine
Taq	*Thermus aquaticus*
Tm	Melting temperature
U	Uracil
UV	Ultraviolet

WSSV	White spot syndrome virus
x g	Centrifugal force

AMINO ACID ABBREVIATIONS

	One letter code	Three letter code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cystein	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

CHAPTER 1

INTRODUCTION

Aquaculture including shrimp farming has been implicated in providing an important source of protein for consumption of the fast growing population on the earth (Subasinghe *et al*., 2009, Stentiford *et al*., 2012). Giant river prawn also known as *Macrobrachium rosenbergii* (or scampi) (De Man, 1879) is a greenish gray and cultivated palaemonidae which is implicated in having a major role in shrimp and aquaculture sector. The modern production of this palaemonidae began in 1960s when the brackish water was found by an FAO expert to be a key factor for survival of the prawn larvae (New and Valenti, 2009).

According to newly published reports, Hawaii, Malaysia, Thailand, India, Vietnam, China and Ecuador have been introduced as well-improved and major contributors to shrimp production in terms of both quantity and value. *M. rosenbergii* is the largest omnivorous palaemonidae. This feeding habit plus other specific traits such as high meat quality and rapid growth have been the key factors for this species to obtain a spectacular commercial value (New, 2002).

M. rosenbergii has been also known as the most popular cultivated shrimp especially in Malaysia and has experienced an exponential increase in production scale since 1970. Historically, increased incidence of diseases among marine and shellfish cultures including crustaceans has emerged to be without doubt a significant impediment for aquaculture and economic development (Soonthornchai *et al*., 2010).

The major bacterial disease spreading among farmed shrimp have been known as necrotizing hepatopancreatitis (NHP) and *vibrio*sis which are respectively caused by *Vibrio parahaemolyticus* (pleomorphic and Gram negative) and several strains of the

genus *Vibrio*. *V. parahaemolyticus* has been also identified as a significant human pathogen in the world (Wong *et al*., 2000).

Shrimp population is in danger of being attacked by various types of *vibrio* like *V. harveyi*, *V. costicola*, *V. splendidus*, *V. alginolyticus*, and *V. parahaemolyticus* at any stage of life which as a result can lead to blood infection (septicaemia) or localized infection in digestive (stomach and hepatopancreas) and non-digestive organs such as heart, gill and lymphoid organs (Panchayuthapani, 1997).

Exploration of immune system in decapods has led to the identification of cellular and non-cellular (humoral) mechanisms based on a self/nonself recognition system. To date, no experimental studies have been reported which are known as adaptive components in association with the invertebrate immune system. This non-adaptive nature of the decapod immune system has made them become exclusively attractive study systems for a better conception of the innate immune system and its response to challenges in various environmental conditions (Mydlarz, 2006).

Clottable proteins -also called coagulogen- through an immune process named coagulation, immobilize the invading pathogens and block their spread throughout the host body. This action also favors the prevention of hemolymph loss in case of exoskeleton injury. Another role of clotting protein has been introduced in crustaceans as being a major factor in recognition and also neutralization of foreign particles (Meng-Yi *et al*., 2005).

Since poor understanding of immune system is available, sustainable production of *Macrobrachium rosenbergii* in large commercial scale takes extensive research on key mechanisms in shrimp immune system especially the clotting process to develop effective control disease strategies in prawn culturing.

Hence, the ultimate purpose of the current study was to characterize the clotting protein gene and to investigate the differential expression of the clotting protein gene (CP gene) associated with the immune system of *Macrobrachium rosenbergii* and its response to *Vibrio parahaemolyticus* infection.

CHAPTER 2

LITERATURE REVIEW

2.1. The Background of *Macrobrachium rosenbergii*

Freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879), has been recognized as the largest palaemonid ever known in the world. The length of the body can reach up to 320mm and 250 mm respectively in males and females (Cowles, 1914; Holthuis, 1950, 2000). Modern aquaculture of freshwater prawn traces its history back into 1960s when work of two pioneers Shao-Wen Ling and Takuji Fujimura and their collaborates led to a breakthrough in mass-rearing techniques in Malaysia and made the production of fresh water prawn possible in commercial scale hatchery (Fujimura and Okamoto, 1972). The first milestone was achieved with recognition of salinity as a key to larval survival by Shao-Wen Ling (Ling & Merican, 1961; Ling, 1977; Ling & Costello, 1979) and the second one was work of Takuji Fujimura and his team which resulted in commercializing the prawn culture with help of advanced farming techniques (New, 2000). These achievements made prawn aquaculture become significant leading to a fast expanding industry in most countries such as Malaysia, Thailand and Taiwan. Over the last decade, the estimated production ratio of *M. rosenbergii* has shown 9-35.48% growth in quantity and 19.68–24.5% growth in value (FAO, 2007) that made this prawn to be included as one of major aquaculture contributor around the world.

2.1.1. Biology of *Macrobrachium rosenbergii*

Macrobrachium rosenbergii can be easily differentiated from other genus' members due to its appearance. These include the existence of a hepatic spine, non-existence of the supraorbital and branchiostegal spines and the easy dactylus of the last three legs. In addition to the prior**,** the rostrum is well improved with teeth

present on the top and bottom (Holthuis, 2000).

Some special features such as high salinity tolerance have made *M. rosenbergii* to be recognized as the most resistant species in comparison to others in genera of *Macrobrachium.* Different salt concentrations of water are easily bearable for *Macrobrachium rosenbergii*. Therefore, they are known as euryhaline animals. This species is of highly commercial interest for its rapid growth with males showing higher growth ratio in comparison to females. *M. rosenbergii* is also able to utilize plants (coconuts, peas, wheat, beans) and animal materials (internal organs of crustaceans, fish and molluscs) omnivorously (New and Valenty, 2000).

Prawns are nocturnal in nature whereby their sense of smell and touch help food materials to be located and thoracic legs are used to grab them. Male prawns are notably bigger than females with also larger and thicker thoracic legs (second pair). Freshwater prawn possesses a big cephalothorax (head) and its long and thick peripods are of equal length appearing clawed. Adult animals appear in blue predominantly and rarely in brown. The wider abdomen together with the long pleura form females a brood chamber to carry eggs till spawning. The male has its genital pores between its bases of the fifth pairs of peripods while those of females can be seen at the base of third peripods. The egg mass is orange before becoming eyed. By the time they grow they appear to be more grayish (Abramo and Brunson, 1996). The summarized morphology of *M. rosenbergii* is given in Figure 2.1.

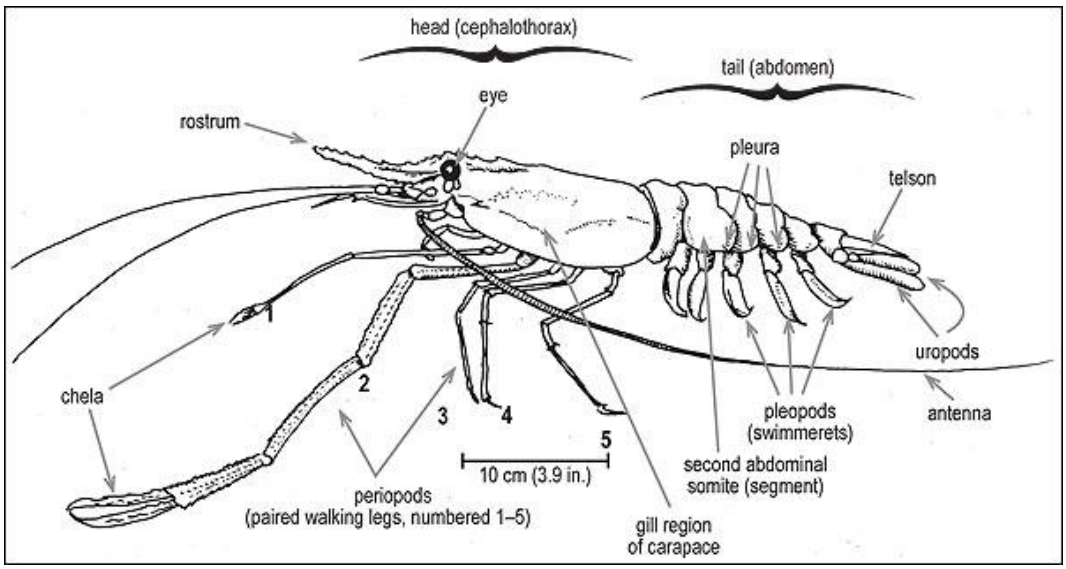


Figure 2.1. : Giant freshwater prawn. External body anatomy according to Forster and Wickins 1972 (New and Singholka, 1985).

2.1.2. Nomenclature and Taxonomy:

Macrobrachium rosenbergii, also known as Malaysian prawn, belongs to the genus *Macrobrachium* and is one of the freshwater prawn species found in tropical and subtropical regions. *M. rosenbergii* has been also described as the largest sized prawn in *Palaemonid* family. Though more than 220 freshwater inhabitant species have been described in the genus *Macrobrachium*, the major numbers of aquaculture experiments and associated researches have been conducted with *rosenbergii* species due to their worldwide commercial interest. The taxonomic definition of *Macrobrachium rosenbergii* is following:

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Infraorder: Caridea

Family: Palaemonidae

Genus: *Macrobrachium*

Species: *M .rosenbergii*

Due to existence of more than 15 synonyms to the name *Macrobrachium rosenbergii,* some big controversies were aroused as to whether *Macrobrachium rosenbergii* has some subspecies or all belong to one species. According to a revision done by Johnson in 1973, two morphological distinct subspecies were identified as *Macrobrachium rosenbergii* schenkeli (Johnson, 1973) and *Macrobrachium rosenbergii* (de Man, 1879) which have been known respectfully as western and eastern freshwater prawn. Later on further morphometric work and allozym studies done in the 1980s following by advanced mitochondrial DNA researches and microsatellite studies in recent years helped to corroborate this result. Revealing a sharp division between two subspecies by microsatellite studies, introduced the name *M. rosenbergii dacqueti* (Sunier, 1925), as the correct name for the western subspecies (Ng and Wowor, 2007).

2.1.3. Life Cycle and Feeding Habit:

Malaysia giant river prawn, *Macrobrachium rosenbergii* inhabit mostly in tropical and subtropical environments. They are amphidromous, whereby the larval development process requires saline conditions. However once the initial stages are passed, they spend most of their life in a turbid condition (Ling and Merican, 1961; Sandifer *et al*., 1975).

Based on recent studies, *M. rosenbergii* and hemimetabolous insects exhibit similar processes in their larval development stages and metamorphosis. The common type of larval development in *M. rosenbergii* include 11 distinct stages with notable and significant growth rate in carapace followed by an allometric growth limited to some specific parts of the body (excluding the carapace) during metamorphosis leading to post larva stage (Shailender *et al*. 2013). Most *Macrobrachium* species prefer clear conditions to live in except *M. rosenbergii* which is usually seen in turbid water (New and Singholka, 1985). Freshwater prawn are not tolerant to low temperature limits the production of *M. rosenbergii* in some geographic areas with cold climates.

The body of freshwater prawn is covered by a hard outer shell which needs to be shed periodically to let the size of the body increase in increment. This process -which is called molting- makes the process of growth divided into 4 distinct stages named as follows (Gaxiola *et al*. 2005):

- Egg
- Larvae
- Post larvae
- Adult

Female prawns with soft shell which just finished their pre-mating molt are ready to mate with hard-shelled males. After mating, a few hour time period is needed for eggs to be laid and also a clean and aerated place is needed for them to be located in. Therefore, eggs will be transferred to somewhere underside of the female's tail. Based on several records, a female in its first spawns can lay at most 20,000 eggs while this number can reach up to 100,000 eggs once the prawn gets mature. After spawning, it takes around 3 weeks for eggs to hatch and get detached from the female's abdomen. In the meantime, eggs -which in primary stages appear in bright yellow- start to change color and become brown and gray-black (Ismael and New, 2000).

The transformation of larvae to post-larvae takes around 11 molts in a period less than 40 days effected by several environmental factors such as temperature, water and food quality and quantity. According to information available about prawn feeding habit, aquatic invertebrates in their initial larval stages and also zooplanktons are known as the major feeding source for *M. rosenbergii* in its larval stage (Sick and Beauty, 1974; Kulum and Ku, 2005). Once the larval stage passes, the availability of turbid water becomes more crucial since the level of salinity tolerance decreases. This transformation happens while its diet is becoming broadened to the point where they can be cannibalistic under certain conditions and food limitations (Kutty *et al*. 2000).

The summery of freshwater prawn life cycle has been illustrated in figure 2.2.

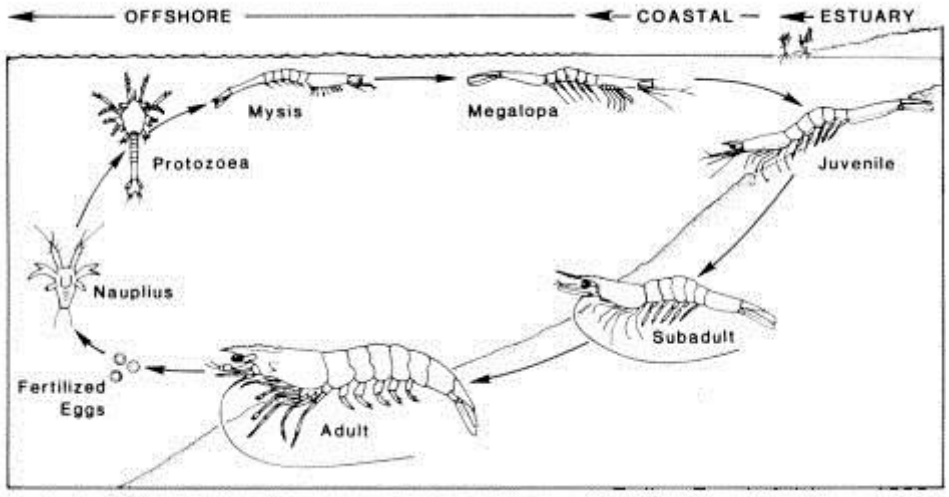


Figure 2.2. Summarized life cycle of *Macrobrachium rosenbergii* (Bailey-Brock and Moss, 1992).

2.1.4. Optimum Salinity Level of Water and Sex Ratio:

The optimum ratio of sex in order to maximize oviposition in *M. rosenbergii* has been recommended through various studies. The male and female ratios of 1:4, 1:3 or 1:5 have been suggested by Melacha (1983),Verghese *et al*. (1992), Suresh Kumar and Kuroup (1998). Moreover, recent experiments done by Ratnayake and his collaborators in 2011 revealed that 1:5 ratio of male and female can be used to obtain remarkably higher larvae while 1:10 ratio gave the minimum number of *M. rosenbergii* seeds and larvae in hatcheries under conditions like those in Sri Lanka. According to another experiment conducted simultaneously, the salinity level of the water in which gravid females led to higher spawning was determined as 5 ppt under Sri Lankan conditions (New and Valenti, 2000; Ratnayake *et al*., 2011)

2.1.5. Dietary Carbohydrates

Several studies on digestive capability of freshwater prawn have shown that feeding with polysaccharides such as dextrin, potato starch and alpha starch can result in a better digestion and also higher rate of growth, however, no fixed carbohydrate diet has been specifically determined for *M .rosenbergii (*Gaxiola *et al*. 2005).

2.1.6. Molting

The body of arthropods is coated by a hardened exoskeleton made of a specific protein called chitin. Since this integument causes a limitation for animal growth, the shell needs to be shattered through a process called molting. This process provides the body with enough space for accumulated and grown tissues. Once the prawn obtains the new elastic shell fully developed, the animal starts to feel quiet and try to get secluded from the crowd till the old coating gets shattered and completely shed off. Softness of the new shell leaves the animal assailable and helpless against predators (Kulum and Ku, 2005).

2.1.7. Mating

Though mating in male sex can take place at any time as long as they are sexually mature, females need to complete a stage called pre-spawning or pre-mating molt which occurs shortly before spawning to get ready to respond (Kutty *et al*. 2000). Several studies done surrounding the process of molting in freshwater prawn has shown that the frequency of molting has an inverse relationship with age. Apart from age, there have been some environmental factors recognized with notable impact on this process such as the quality and availability of food and water source. Newly molted prawn is vulnerable at the beginning and it takes some time (ordinarily around 2-6 hours) to obtain a sufficiently hard and vigorous shell to predation. The next molt usually occurs after a time period of 20-40 days depending on associated factors mentioned before.

Females get held between thoracic legs of the desired males and then sexual intercourse happens during less than a minute. A specific part of thoracic legs on the ventral region is carrying a mass of sperms covered by a soft gelatinous layer to protect sperms and keep their integrity (Ismael and New (2000).

2.1.8. Prawn Hatchery

Newly advanced technologies in prawn rearing systems and larval culture techniques have enabled this industry to produce post larvae in big quantities and commercial scales in most of tropical and subtropical regions such as Malaysia. Over the past few years, the cost of clear water transportation in prawn hatcheries has emerged to be an issue in raising freshwater prawn in producing countries. Since majority of hatcheries use transported sea water for their prawn culture, the further the hatchery is from the sea, the more the operational cost would be. Therefore it takes good operational management skills to minimize the risk and ensure a steady production of post-larvae in this industrial sector (Shailender *et al*. 2013).

2.1.9. History of global status

Prawn or shrimp aquaculture constitutes a primary resource of human livelihood in more than 70 countries and has been known as an important nutritious commodity with high economic impact. Among these 70 countries, Ecuador, Indonesia, China, Thailand, Vietnam and India have surpassed other countries at shrimp farming and aquaculture with around 80% of global shrimp production (Dastidar *et al*., 2013).

2.1.10. Commerce and Shrimp Production

There are more than 50 varieties of prawns widely distributed in marine waters with either temperate or tropical ecosystems. Among all these 50, only 15 species are economically important (Wyban, 2009). Some specificities such as good taste, high

protein and vitamin content, omega 3 fatty acids, anti-oxidants, essential minerals and selenium have made 5 species − Giant freshwater prawn (*Macrobrachium rosenbergii),* tiger shrimp (*Penaeus mondon),* white leg shrimp (*Penaeus vannamei*), Atlantic-white shrimp (*Penaeus setiferus)* and Indian-white shrimp (*Penaeus indicus)*− become the most popular nutrient and commodity in inland fisheries sectors (Lifestyle Lounge : Health & Fitness, 2012; FAO, 2012).

Based on studies carried out by Prabir G. Dastidar and Ajoy Mallik in 2013, prawn production and research contribution were introduced as two primary factors to categorize major countries involved in prawn aquaculture. High production and research contribution protect associated countries against the risk of economic loss while weakness in these two factors can be considered as a major threat to sustainable production of shrimp. Therefore such countries would encounter a gigantic risk of financial losses due to any disease outbreaks caused by infectious microorganisms (Dastidar *et al*., 2013).

According to the recently released data by FAO Fisheries and Aquaculture Department in 2013, the contribution of aquaculture to the global production of seafood represents an average growth rate exceeding 6.2 percent per year since 2001 and the farmed aquatic production reached near 63 million tonnes in 2011. However a negative growth was experienced by some global major producers such as Malaysia, , USA, Myanmar and Japan due to the combined impact of infectious disease and weather associated factors such as floods, the total aquaculture output enjoyed a positive growth for the years 2011, 2012 and 2013 (FAO, 2013).

Table 2.1 presents estimates for trend outlook of shrimp aquaculture in major producing nations for the years 2011-2013 based on production form - green/head on/head off, pealed, cooked and breaded (Valderrama and Anderson, 2012).

Table 2.1. Expected trends in shrimp aquaculture – GOAL survey 2011.

Product form	Asia	America	World
Green/Head-on	Stable	Stable/Increase	Stable
Green/Head-off	Stable	Decrease	Stable/Decrease
Pealed	Stable	Increase	Increase
Cooked	Stable	Increase	Increase
Breaded	Stable	Increase	Stable/Increase
Other forms	Stable	Increase	Stable

As projected in Figure 2.3., China, Thailand, Vietnam, Indonesia, India and Bangladesh have been polled by FAO and GOAL in 2011 as top 6 Asian producers in farmed shrimp aquaculture. Among these 6 nations, China remains at the highest level of shrimp production since 2006 and it is also predicted to grow in 2014 in comparison to the production during the years 2012 and 2013.

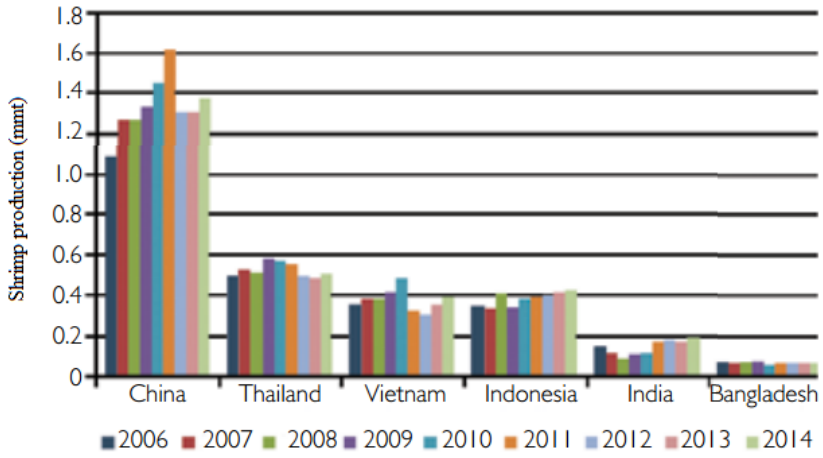


Figure 2.3. Cultured shrimp production in major producing countries in Asia. (GOAL, 2012).

2.1.11. Constraints

Nevertheless market familiarity of M. *rosenbergii* has made it recognized as a major consumption source, there have been some real or perceived constraints inhibiting the global farmed shrimp production as follows:

It is not possible for *M. rosenbergii* to be reared in a grow-out system. Moreover, its larval cycle takes as twice as the time in other marine shrimps. However it used to be a negative point in the past researches, it has been recently regarded as an advantage because of some sustainability reasons. Cultured *M. rosenbergii* like any other commodities is under market and economic pressure due to the competition exposed by cheap imported shrimps from other nations. Hence the difference between local and imported shrimp price has made a serious deal for individual producers. Moreover this prawn requires more pre-cautions and cares in processing procedures than marine shrimps.

As demonstrated in Figure 2.4. some issues and challenges involved in global production of farm-raised shrimp have been investigated in a survey published by GOAL in 2011. According to that, there are more than 15 factors threatening aquaculture growth and among all of them, disease has been emerged as the main constraint to global production of shrimp. After disease outbreaks, production cost, international market prices, seed stock quality and availability, access to disease-free broodstock and product quality control are another 5 major factors impact the sustainability of shrimp aquaculture industry the most. These characteristics have made the aquaculture to become a high risk industry suffering from huge financial losses every year. These issues can be overcome and managed only by research and development (GOAL, 2011)

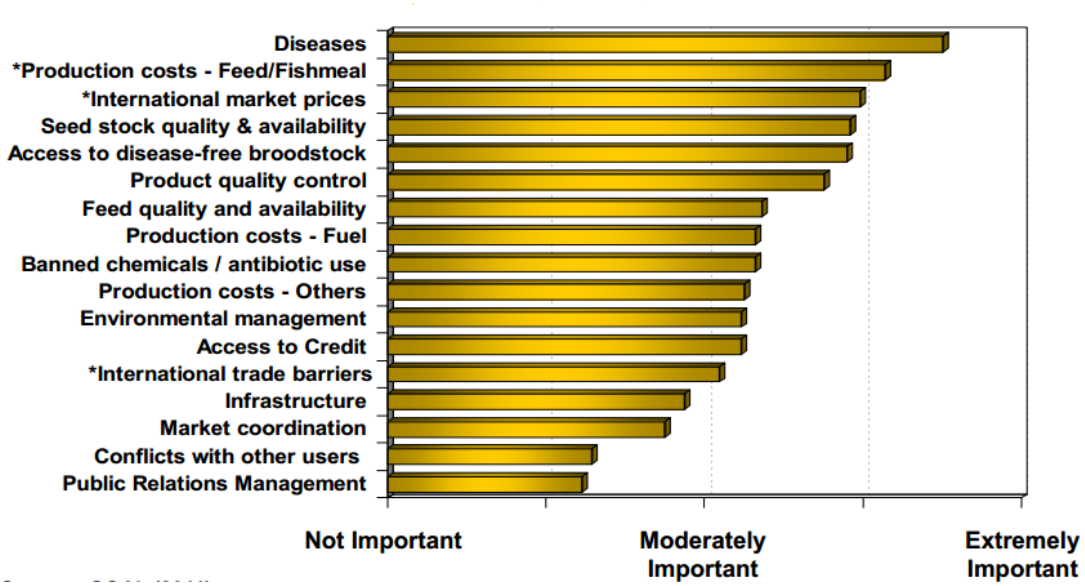


Figure 2.4. Issues and challenges associated with global shrimp production (GOAL, 2011). Top 3 issues are shown by asterisk.

2.1.12. Prognosis

Taking all negative and positive points of freshwater prawn into consideration, an extremely bright future has been expected for the global market of *M. rosenbergii*; however, further advancements and more improved culturing techniques are needed to exploit potential opportunities for a notable expansion in aquaculture sector.

2.2. *Vibrio parahaemolyticus*

2.2.1. *V. parahaemolyticus* Infection in Respect to Shrimp Aquaculture and The Supply Chain:

The majority of newly done reports have been to identify *vibrios* as a major cause for shrimp infection and setback of aquaculture. Some organs such as hepatopancreas, gill and feed have been reported as the most probable routes of primary infection (Austin *et al*., 2005). These bacteria get the host tissue colonized once they manage to pass through epithelial cells. Although yet, there is a lack of simply comprehensive and

trustworthy model for a better conception of virulence potential and mechanism of bacterial interaction (Chatterjee and Haldar, 2012).

2.2.2. Back Ground of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus, a Gram-negative halophilic bacterium has a curved and rod shape with single polar flagellum. This species is motile like other species of genus *Vibrio*. *V. parahaemolyticus* is considered as facultative aerobic and non-spore forming organisms (DePaola *et al*., 2000; Robert-Pillot *et al*., 2010).

2.2.3. Habit and Source of *V. parahaemolyticus*

Since this bacterium is categorized as salt required organism, it is found in brackish salt water. It inhabits in warm coastal and marine environment (Johnson *et al*., 2009).

2.2.4. Classification and Taxonomy:

Kingdome	Bacteria
Phylum	Proteobacteria
Class	Gamma proteobacteria
Order	*Vibrio ales*
Family	*Vibrionaceae*
Genus	*Vibrio*
Species	*V. parahaemolyticus*

(Fujino *et al*., 1951; Sakazaki *et al*., 1963)

Figure 2.5. *Vibrio parahaemolyticus*, the global cause of gastroenteritis (Makino *et al*., 2003).

2.2.5. Cell and Colony Morphology of *Vibrio*

Vibrio parahaemolyticus contains numerous cell types resulted in various capabilities which help them to survive under different circumstances. Swimmer, Swarmer, Opaque and Translucent cells are different cell type of *V. parahaemolyticus* which help bacteria to modulate their functions in order to adaptation and survival in changing environment (McCarter, 1999). *V. parahaemolyticus* colonies can be easily identified and distinguished by their unique purple color (Eddabra *et al*., 2011)

Figure 2.6. Purple colonies of *Vibrio parahaemolycus* grown on chromID after over-night incubation at 37 °C (Eddabra *et al*., 2011).

2.2.6. Disease and Epidemiology

Over the past 4 years, shrimp production sector faced a significant setback due to sudden prevalence of a deadly bacterial disease called Early Mortality Syndrome (EMS) or with a more descriptive name Acute Hepatopancreatic Necrosis Syndrom (AHPNS) in Southeast Asia. The disease symptoms include abnormality in hepatopancreas (discolored and shrunken), swimming in a corkscrew manner and also loose shells. EMS infection makes them become slow growers with pale coloration. The infected shrimps then sink to die in depth of the pond. The causative agent of the previously talked disease had been a mystery since 2009 –when it first surfaced Chinese market-until last few months. Precise investigation and laboratorial experiments carried out by a group of experts and scientists succeeded in identification of causative pathogen of early mortality syndrome (EMS) or more technically, acute hepatopancreas necrosis syndrome (AHPNS). *Vibrio parahaemolyticus* was finally introduced as the micro-

organism causing EMS and was identified and isolated by scientists in 2013 (Tran *et al*., 2013).

It has been of interest to know that those strains of *vibrio* which result in death in shrimp are not considered as a threat to human health. But consumption of seafood contaminated with those strains of *vibrio* which threaten humans result in gastroenteritis (Wong *et al*., 2000). Consumption of raw or undercooked sea-born food contaminated with bacteria has been recognized as the most probable fecal oral route for ingestion of *V. parahaemolyticus* rather than wound infection. In subsequent, in 60% to 80% of infections, acute gastroenteritis and diarrhea occur within only 24 hours after consumption and in more severe cases it is accompanied by other symptoms such as vomiting, nausea, abdominal pain and fever which most often last for 2-8 days. Such infection leads seldom to serious disease unless in immunocompromised individuals (people who are suffering from weakened immune system).

Ear and eye infections are other organs in danger of getting infected as a result of long swimming in warm waters with high concentration of *V. parahaemolyticus.* These bacteria can be also causative of skin problems due to exposure of an open wound to seawater (Altekruse *et al*., 2000; Venkitanarayanan and Doyle, 2001). Prevalence mostly occurs during the warm months when the concentration of bacteria gets higher due to water temperature of coastal area which becomes warmer resulting in sufficient environment for bacteria to increase up to infection levels (Daniels *et al*., 2000).

2.2.7. Treatment in Shrimp

Applying a good management for sanitation and rigorous water preparation can be the initial steps to help reduce the level of stress on shrimp and block possible entry of *vibrio*sis to the culture water (Baticados *et al*., 1990). It is also of high importance to apply appropriate partial harvesting techniques to reduce pond biomass and increase

pond water exchange. Furthermore, Lime/dolomite administration, draining after harvesting and good pond designing are other measures to take for a notable reduction in level of mortality caused by this disease (Nash *et al*., 1992).

2.2.8. Treatment in Human

In majority of cases the poisoning caused by *V. parahaemolyticus* is self-limiting. The effectiveness of antibiotic action in treatment of *vibrio* infected individuals has not been proven yet. While, drinking plenty amount of water and liquids is highly recommended in order for lost body fluids to be replaced and the severity of symptoms get reduced. Although, some antibiotics like tetracycline or ciprofloxacin may be prescribed by the specialists as a cure for prolonged disease. In these cases it is of high importance to take the antimicrobial susceptibility feature of the organism into consideration (Baker-Austin, 2013).

2.2.9. Virulence of *V. Parahaemolyticus*

Previously done researches have introduced a hemolytic toxin named KP+ factor responsible for *vibrio* poisoning symptoms. Shiga-like toxin is another probable virulence factor yet to be studied (Desmarchelier, 1997).

There are three major pathogenicity proteins of *V. parahaemolyticus*. Thermostable direct hemolysin (TDH) is considered as the most significant virulence factor due to its infective activities. In those strains of *V. parahaemolyticus* with deficiency of TDH, the TDH-related hemolysin (TRH) is account for the virulence. The presence of these two hemolysin genes in any strains is a proven reason of pathogenicity in *Vibrio parahaemolyticus*. (Robert-Pillot *et al*., 2004). Two groups of genes for type III secretion systems located on chromosome 1 and 2 are recognized. $T3SS_1$ and $T3SS_2$ are responsible for cytolytic and enterotoxicity activities in bacterium respectively (Park *et al*., 2004).

2.3. Immune System in Crustaceans

Crustaceans have been able to show a spectacular adaption to aquaculture and have manifested themselves as a group of economic relevance, however, emergent disease and infections have been threatening this industrial sector by causing notable economic losses and disease transmission from marine culture to natural population (Ward and Lafferty 2004; Little *et al*. 2005).

Once effective factors such as environmental change and new potential pathogens overcome the host resistance barrier (the first line of invertebrate defense), they may finally result in disease out breaks and economic losses (Harvell *et al*. 2004, Lafferty *et al*. 2004, McCallum *et al*. 2004). To date, the model system introduced for invertebrates, suggest a non-adaptive immune system -known as a vertebrate immunity precursor- categorized in 3 distinct mechanisms defining the efficiency of an immune response:

First of all, the organism tries to discern between self and non-self. Secondly, a defensive response with the aim of inactivation or elimination of invader triggers; and at the end, some processes are taken for recognition of damaged or infected cells and attempts are made to eliminate them. These primary actions are associated with three essential components involved in innate immune system (Midlarz *et al*. 2006):

- Phagocytosis which is identified as a cell-mediated process.
- Humoral response activation which results in some cell-free processes listed as opsonization, coagulation and melanization.
- Another cell-free process leading to production of some antimicrobial and humoral compounds.

According to the result of some experiments done with the aim of identifying evidences on defense factors being antigen-specific, some cellular and humoral factors have

shown to be capable of recognition of damaged and non-self cells with high and considerable specificity. Studying invertebrate immune system, physical barrier has been known as the first impediment to pathogenic invasion. Once this barrier is broken, immune system triggers proteolytic pathways aimed at diminution and clearance of invading pathogens (Janeway and Medzhitov, 2002).

Coagulation cascade is a key mechanism in invertebrate immune defense aiming to avoid hemolymph loss. This process also activates a concatenate enzymatic reaction – proPO system – which leads to production of black-brownish pigments called melanin. Stimulation of oxidative metabolites has been introduced as another result of coagulation cascade. Other effector mechanisms such as encapsulation, phagocytosis and nodule formation take place following activation of prophenoloxidase. The mechanisms triggering these processes are highly associated with the function of specific proteins termed pattern recognition receptors identifying glycosylated pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002).

2.3.1. Recognition Molecules

Immunoglobulins are soluble or cell surface molecules identified in immune system of higher animals which are highly involved in specific recognition process of cells. However defense mechanisms of invertebrates lack such feature, they do include specific structured proteins conceptually termed as pattern recognition proteins (PRPs) which recognize the highly conserved signature molecules on the surface of pathogenic intruders (Luo *et al*., 2006).

Studies and experiments conducted on innate immune system of crustaceans have resulted in identification and characterization of numbers of pattern recognition molecules such as LPS-binding proteins – recognition of lipopolysaccharides on bacterial cell walls- (Sun *et al*., 1990, Natori and Kubo, 1996; Lee *et al*., 1996;

Dimopoulos *et al*., 1997), b-1,3-glucan binding proteins (BGBP) - recognition of signature molecules on fungal cell walls- (Ochiai and Ashida, 1988; So¨derh¨all *et al*., 1988; Duvic and So¨derh¨all, 1990, 1993; Seki *et al*., 1994), and Peptidoglycan-binding proteins – identification of PGNs on bacterial cell walls- (Yoshida *et al*., 1996; Kang *et al*., 1998; Ochiai and Ashida, 1999). These interactions take part instrumentally in activation of highly important pathways and key molecules.

Another class of PRPs has been represented as lectin-like proteins described in crustaceans. Among several types of lectin-like proteins, type C has been identified to have at least one recognition domain for carbohydrates (CRD). This protein highly maintains its well-defined structure with the help of a few pairs of disulfide bonds (Liu *et al*. 2011). Having studied different groups of lectins in penaid shrimps infected by WSSV and Gram-negative bacteria, the infection resulted in up-regulated expression of lectin in organs such as hepatopancreas and hemocytes (Luo *et al*., 2003; Ma *et al*., 2007; Yang *et al*., 2007).

2.3.2. Prophenoloxidase System in Immune System of Crustaceans

Having an open circulatory system and lack of adaptivity in immune system of invertebrates in comparison to those of vertebrates, make the survival of animal highly depend on how immediate and effective the intruders get recognized and immobilized in a well-elaborate manner (Soderhall, 1982; Ratcliffe *et al*., 1985). Clotting system is known as a candidate for prevention of blood loss and proPO activating system is identified as an instant non-inducible system in innate defense mechanism (Soderhall *et al*., 1990).

A major part of innate immune system in crustaceans lies in several concatenated enzymatic reactions representing high resemblance to complement system (Sritunyalucksana and Söderhäll, 2000). This momentous process which is in high control of enzyme phenoloxidase (PO) is accompanied by melanization of invasive

microorganisms entering the hemocoel for inactivating them and avoiding spread of foreign particles through host body. Moreover, this process helps to heal cuticle damage (Cammarata and Parrinello, 2009). There have been several studies conducted with the aim of elucidation of mechanisms through which zymogenic proPO turns into its active status in the presence of Ca^2+. Contacts and stimulation of cells with LPS or β-glucans and other versions of pathogen associated molecular patterns (PAMP) make the prophenoloxidase activation take place requiring serine protease cleavage. Phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1) is a copper-dependant enzyme that plays a key role in production of a pigment ubiquitous called melanin found in animal kingdom (Amparyup *et al*., 2009). O-hydroxylation of monophenols (cresolase activity) and the succedent oxidation of the O-diphenols to quinines are related to PO (Kahn, 1985; Sugumaran *et al*., 1988; Cammarata and Parrinello, 2009).

2.3.3. Phagocytosis

Studies on non-specific immune system showed that if foreign particles or micro-organisms such as spores and bacteria were introduced into an invertebrate host, some of them were phagocytized and ingested in a non-specific manner by a group of cells called hemocytes or phagocytes (Iwanaga and Lee, 2005). This process -which belongs to early evolutionary stage of life - has been recognized as an essential reaction for fighting infections and the most common process for subsequent immune response (Sierra *et al*., 2001). Phagocytosis comprises unique-featured cells which are capable of ingesting and destroying harmful and invasive microbes and also senile (modified) and dead cells of the own body ,however, some harmful micro-organisms have evolved methods to come over this immune reaction and get to multiply intracellularly resulting in a symbiotic state or death of the host (Soria e al., 2006; Mun˜oz *et al*., 2000).

Having performed several in-vitro studies on phagocytic activity, the flow cytometric evaluation showed that the immune stimulation in freshwater prawn, *Macrobrachium rosenbergii* and *Penaeus mondon* is followed by production of ROS or reactive oxygen species (Mori and Stewart 2006; Jiang *et al*., 2006).

2.3.4. Encapsulation

Encapsulation is a common immunity mechanism comprises multicellular action forming multilayered hemocytic capsule around foreign particles and parasites which humoral defense is unable to destroy. This action can either eliminate or kill the intruders or lead to restriction of its movement and growth (Persson *et al*., 1987).

Theories advanced on composition of a typical capsule suggested that it consists of 5-30 layers of hemocytes tightly compact with no intracellular space that can also act as a protective barrier in case of cuticle damage which as a result protects muscle and inner organs against entrance of offending pathogens (So¨derha¨ll *et al*. 1984). Histochemical analysis has also revealed the acidic or neutral compounds such as glycoproteins and mucopolysaccharides in hemocytes. 3 factors known as the causes of destruction in encapsulated particle are as follows: hydrolysis action, production of quinons and oxygen reduction (Rather and Vinson, 1983; Vazquez *et al*., 2009).

2.3.5. Clotting Protein

Clottable proteins or coagulogens are known as critical components in crustacean immune response against microbial invasion and are implicated in an important immune process called coagulation. This mechanism effectively blocks the access and propagation of pathogen in other parts of the body and in case of exoskeleton injury it favors the host survival by preventing loss of hemolymph (Meng-Yi *et al*., 2005). Recent studies have suggested that clotting protein is associated with other defense mechanisms such recognition and neutralization of infectious particles.

According to the theories advanced on clotting mechanism in crustaceans, 3 types of cascades are represented as detailed below:

- In type A, hemocyte agglutination occurs rapidly with no presence of plasma coagulation.
- In type B, cellular aggregation observed to happen while there is a limited coagulation in plasma.
- In type C, which is the known as the coagulation type in shrimp, plasma coagulation occurs after a limited state of cellular aggregation (Yeh *et al*., 1999; Van de Braak, 2002).

Hemolymph plasma is where Clotting protein exist the most, more than in hemocytes. Once immune system is stimulated by the microbial cell wall components, it proceeds to coagulation mechanism which is recognized as a proteolytic cascade. Then, hemocytes start to produce and secret transglutaminase which is a Ca^{++} dependent enzyme and is capable of forming a covalent cross-link between the side chains of free glutamine and lysine residues of clotting proteins and form a large aggregate (Fig. 2.7.) (Wang *et al*., 2001b). In this process, particular molecules on hemocyte membrane - known as membrane specific lipopolysaccharide receptors- play a key role in detection of bacterial LPS molecules present on their surface (Ariki *et al*., 2004).

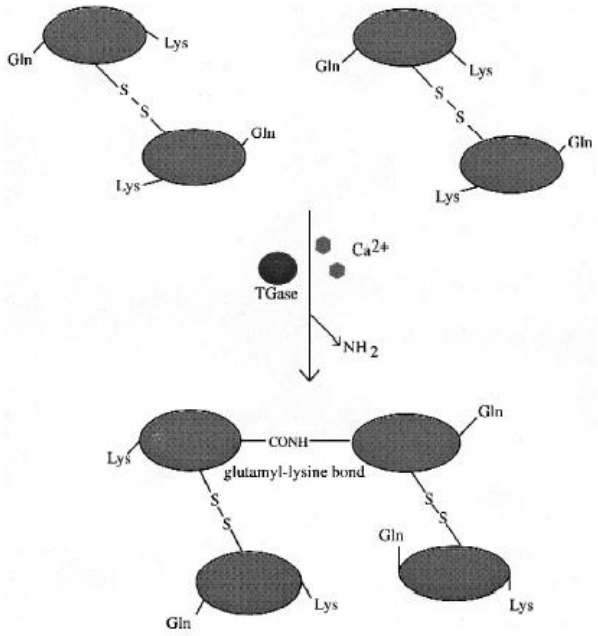


Figure 2.7. The clotting mechanism introduced in crayfish. The 2 subunits of CP – 210 KDa per subunit – are linked by disulfide bond. Transglutaminase enzyme favors the cross-linking of this dimeric protein in presence of Ca^{++} (Wang *et al*., 2001b).

Several studies surrounding crustacean innate immune defense, have identified the clottable proteins in some species such as *Ibacus ciliates (*Komatsu and Ando, 1998), *P. monodon* (Yeh *et al*., 1998) and the freshwater crayfish (Kopa´cek *et al*., 1993)

Clotting proteins are known as homodimeric structured glycoproteins with a molecular weight of approximately 380-400 KDa. There have been also some similarities recognized in composition of amino acids and some N-terminal sequences.

Table 2.2. A brief comparison of N-terminal sequence alignment in clottable proteins of 4 species: *P. monodon* (Yeh *et al*., 1999), *I. ciliatus* VHDL (Komatsu and Ando, 1998), *P. leniusculus* (Kopa´cek *et al*., 1993), and *P. interruptus* fibrinogen (Doolittle and Riley, 1990). The bold face indicates corresponding residues in clotting protein of shrimp.

P. mondon CP	**L**QPG**LEYQY**K
I. ciliates VHDL	**L**QPG**LEYQY**R
P. leniusculus CP	**L**HSN**LEYQY**R
P. interruptus finbrinogen	**L**QPK**LEYQY**K

In *Tachypleus tridentatus*, some specific zymogenes known as serine protease are involved in hemolymph clotting process as well. Once LPS-specific receptors of hemocytes are activated, serine protease zymogenes subsequently get released. This happens while bacterial LPS and fungal β- 1,3 glucan have the capability of activating serine protease zymogenes in an automatic manner. Such mediated cascade has been also identified in clotting system of horseshoecrab (Tamura *et al*., 1996; Vazquez *et al*., 2009).

CHAPTER 3:

Methodology

3.1. Identification of Putative Clotting Protein Gene in *M. rosenbergii*

The full length putative clotting protein gene was sequenced from transcriptome unigenes from *M. rosenbergii* using Illumina Solexa sequencing technique. Concisely, the short reads obtained from RNA sequencing in certain organs such as hepatopancreas and gill were assembled constructing unigenes and then mined for those sequences that were identified as putative clotting protein gene through NCBI-BLAST homology search (http://blast.ncbi.nlm.nih.gov/Blast).

3.1.1. Sequence Analysis:

The nucleotide sequence of putative clotting protein has been translated to amino acid sequence using ExPASy (Gasteiger *et al*., 2003). Our sequence, putative clotting protein, has been used to check for any closely related sequences in NCBI-BLASTP (Altschul *et al*., 1990) and Uniprot (http://www.uniprot.org/). Sequences with a good percentage of similarity have been selected for further alignments -multiple sequence alignment using ClustalW (Thompson *et al*., 1994) and constructing a phylogenetic tree using using Neighbor-Joining method at MEGA 5.05. (Dereeper *et al*., 2008; Dereeper *et al*., 2010). The DNAstar protein analysis (http://www.dnastar.com/) has been done on *M. rosenbergii* putative CP and analysis using PROSITE has been conducted to identify possible domains on this protein (Sigrist *et al*., 2002). Phyre 2 server has been used to check the structural similarity of CP with proteins having PDB structures (Kelley and Sternberg, 2009). Identification of signal peptide cleavage site for putative clotting protein has been done using Signal-P worldwide P server (http://www.cbs.dtu.dk).

3.1.2. Collection of Healthy Giant Freshwater Prawn:

Apparently healthy *Macrobrachium rosenbergii* prawns with the average body weight of 25.1±4.8 g and the body length of 13.4 ±0.8 were collected from a hatchery in Pantai, Negeri Sembilan Darul Khusus, and Malaysia. They were acclimatized in flat bottomed glass tanks (300 L) in Institute of Science Biology of University Malaya. Tanks contained aerated and anti-chlorine treated fresh-water with a controlled temperature of 28 ± 1° C. Prawns were maintained in aquaria for minimum 3 days before being challenged to *Vibrio parahaemolyticus* and PBS. A maximum of 10 prawns per tank were maintained during the experiment and they were fed two times a day by commercial prawn feed.

3.1.3. CP Primer Designing

A primer set was designed (Table 3.1.) for PCR and qPCR using PrimerQuest program (http://www.primerquest.com/) and was optimized using Promega reagent by PCR machine as directed by manufacture's protocol.

Table 3.1. Specific primers for putative CP sequence

Name	Sequence (5' to 3')
CP-F	TCTGTTCTCGTTATCTGCCCGCTT
CP-R	TGACG TGCTCCTGATGTCGTGGGATTCTT

3.1.4. Total RNA Extraction

Total RNA was extracted from gill of apparently healthy prawn using TRIzol Reagent following the provided manufacturer's protocol (Guangzhou Dongsheng Biotech, China) (Appendix A). Determination of RNA concentration extracted from each tissue was done spectrophotometrically using nanodrop.

3.1.5. First Strand cDNA Synthesis

First-strand cDNA was synthesized from total RNA extracts by ImProm-II reverse transcriptase (Promega ImProm-II reverse transcription system) following the manufacturer's guideline with oligo (dt) primers. In order to prepare RNA and primer mix (mix A) for 20 µl final reaction, 100 ng of each RNA sample was added to 1 µl of oligo dt primers and 3 µl of nuclease free water in a final volume of 5µl. This mixture was then incubated in PCR machine for 5 minutes in 70 °C and then was immediately chilled at 4° C for another 5 minutes. The reverse transcriptase mix (mix B) for 20 µl final reaction contained 6.1 µl nuclease free water, 4 µl ImProm-II™ 5X Reaction Buffer, 2.4 µl MgCl2 (25 mM), 1 µl dNTP mix (10 mM), 0.5 µl recombinant RNasin® ribonuclease Inhibitor and 1 µl ImProm-II™ Reverse Transcriptase (in a final volume of 15µl). After a light vortex mix A was added to mix B and reverse transcription PCR was performed to synthesize First-strand cDNA from total RNA. The PCR cycle profile was 5 minute annealing step at 25 °C followed by 60 minute extension at 42°C and 15 minute inactivation at 70°C.

3.1.6. PCR Optimization for Amplification of Putative CP cDNA from Gill of Healthy Prawn

cDNA amplification was performed using Promega reagent through an S1000^{TM} BIO RAD thermal cycler with the aim of primer optimization. Amplification reactions were prepared in total volume of 10 µl including 50 ng cDNA (extracted from gill), 1.5 mM MgCl, 1X Promega Green GoTaq® Buffer, 0.2 mM dNTPs, 0.025 U/µl Promega GoTaq® DNA Polymerase, 0.25 µM forward primer and 0.25 µM reverse primer. The temperature profile was set as follows: 95° C for 5 minutes as an initial denaturation step, 30 cycles of 95°C for 5 minutes (denaturation), a 55 - 65°C annealing step for 1 minute, a 1 minute extension at 72°C followed by a final extension cycle at 72°C for 5 minutes.

Then PCR products were run on gel electrophoresis (agarose gel 1%) using 1X TBE buffer for 30 minutes in 100V followed by a 35 minute gel staining in ethidium bromide. The results were observed by AlphaImager™ Gel Imaging machine (Cell Biosciences, U.S.A).

3.1.7. PCR Clean-up and Primer Verification:

To verify the primers, PCR product was subject to clean-up using NucleoSpin® Extract II PCR clean-up kit according to the manufacture's guideline (Appendix B). The purified PCR product was collected and sequenced by 1st BASE. *(*http://www.base-asia.com/dna_sequencing/*).* Sequencing output was aligned using MEGA5(Tamura *et al*., 2011) to the specific part of cDNA that primers were assumingly designed for.

3.2. Relative Putative CP Gene Expression in Different Tissues of Healthy Giant Fresh water Prawn

3.2.1. Extraction of RNA from Six Various Organs of Healthy *M. rosenbergii:*

Six organs i. e. muscle, gill, stomach, pleopods, hemolymph from heart and hepatopancreas of three apparently healthy *M. rosenbergii* were dissected. A sterilized syringe 29G x ½" was used in order to collect 0.2-0.5 ml hemolymph from heart of each prawn. Hemocytes were then isolated from haemolymph with the aim of total RNA extraction by performing 10 min centrifuge at 3000 X g at 4ºC. Tissues were immediately dropped in liquid nitrogen to be snap-frozen. All samples were then preserved at -80 freezers prior to total RNA isolation. Samples were subject to total RNA extraction following the previously mentioned procedure.

3.2.2. Reverse Transcription for First Strand cDNA Synthesis from Total mRNA Transcript Extracted from Various Tissues:

The conversion of total RNA obtained from six organs to cDNA was conducted using Promega reagent (ImProm-II Reverse Transcription kit) based on the previously described procedure.

3.2.3. Relative Quantification of Putative CP mRNA Expression by qPCR:

a) Primer Designing for Real-Time PCR

In addition to the PCR primer set designed for a part of ORF sequence of putative CP mRNA transcript, another set of primer was designed as controls based on ORF sequence of Elongation Factor -1α (EF-1α) cDNA which is assumed to be constitutively expressed (Bustin, 2002; Bustin *et al*., 2005). The primer designing was done using PrimerQuest software (http://www.primerquest.com/) and verified by sequencing techniques.

Table 3.2. Real-time PCR primer sets for CP and EF-1α

Gene	Primer set	Sequence (5' to 3')	Amplicons size
Putative CP	CP-F	TCTGTTCTCGTTATCTGCCCGCTT	99bp
	CP-R	TGACGTGCTCCTGATGTCGTGGGATTCTT	
EF-1α	EF90F	ACTGCGCTGTGTTGATTGTAGCTG	90bp
	EF90R	ACAACAGTACGTGTTCACGGGTCT	

b) Optimization of Primer Concentrations in Real-Time PCR and Efficiency Determination to Validate Statistical Analysis

Efficiency determination of primers performance (CP and EF-1α in current study) during amplification is of high importance to validate further statistical analysis of relative quantification PCR using the comparative Ct method $2^{-\Delta\Delta Ct}$. For the statistical analysis to be valid, approximately equal primer efficiencies need to be achieved (Livak and Schmittgen, 2001). Real time PCR efficiency is calculated using the formula of efficiency = 10 [–1/slope]-1 (Pfaffl, 2001). The calibration curve of real-time PCR reactions with acceptable efficiencies result in slopes ranging from -3.10 to -3.58 which respectively correlates to 110-90 % amplification efficiency (reactions with 100% efficiency result in a linear slope of -3.32). Accordingly, the result of E value calculation varies between 1.60- 2.10 (Pfaffl and Hageleit, 2001).

A serial dilution was done to prepare five different concentrations of prawn gill cDNA at 50 ng/ µl, 5ng/µl, 0.5ng/µl, 0.05ng/µl and 0.005ng/µl. The real-time PCR using ABI SDS 7500 (Applied Biosystems) was run with amplification reactions at total volume of 15 in each tube including 1X SYBR® Green master mix, 0.6 or 0.3 or 0.15 mM of forward and reverse primers (CP and EF-1α) and 1 µl of 50 ng/ µl, 5ng/µl, 0.5ng/µl, 0.05ng/µl and 0.005ng/µl cDNA with preparation of 3 replicates for each sample tubes. The temperature profile for absolute quantification real-time using ABI SDS 7500 Real-Time PCR System (Applied Biosystems) was set as standard conditions: AmpliTag Gold activation (the first stage) at 95°C for 10 minutes, 40 repeats of 15 second denaturation stage at 95°C, annealing and extension at 60°C for 1 minute followed by dissociation stage for curve analysis. The results were used to build a standard curve for each primer set (CP and EF-1α) between cDNA concentrations on X-axis and the average of Ct value on Y-axis. The m-value was determined according to the curve slope. Primer efficiencies for EF-1α (as an endogenous primer) and CP (as target) were

calculated using the equation E=10 [–1/slope]-1 and the results were compared. Those concentrations which resulted in better standard curve and closest efficiency values were used for further experimental analysis.

c) Tissue Expression Profile Using Real-Time PCR:

Real time PCR was performed in ABI SDS 7500 Real-Time PCR System (Applied Biosystems) reactions with a volume of 15 µl including 1X SYBR® Green PCR master mix, optimized concentration of forward and reverse primers followed by adding 1 µl of 50 ng/µl cDNA of different tissues of healthy prawns. Real-time PCR runs were completed at standard temperature conditions set as follows: AmpliTag Gold activation (the first stage) at 95°C for 10 minutes, 40 repeats of 15 second denaturation stage at 95°C, annealing and extension at 60°C for 1 minute. Real-time fluorescence detection was done by a built-in CCD camera (changed couple device) and data were analyzed using SDS software 1.3.0. To provide highly reliable results and to maintain accuracy of fluorescence measurements the base line was automatically set by the software according to the background signal. The numerical value of threshold cycle (Ct) was measured by SDS software as 10 standard deviations. EF-1α was used to normalize the amplification analysis. The comparative Ct method $2^{-\Delta\Delta Ct}$ was used to determine the CP expression level in each sample tissue (Livak, 2001) and the highest mean ΔCt threshold cycle value was defined as calibrator i. e. the expression level of CP gene in tissues correspondent to those with the lowest ΔCt. Final results were demonstrated as means with S.D.

d) Statistical Analysis of Putative CP Gene Expression Level in Different Organs of Healthy *M. rosenbergii*

Comparison of relative CP expression between gill, hemolymph, hepatopancreas, muscle, pleopods and stomach was statistically analyzed with one-way ANOVA. Turkey's HSD test was performed for mean comparison using IBM SPSS version 20.0 when a 5% significance level was observed.

3.3. Differential Expression of Putative CP in Stomach of *Vibrio* Challenged and PBS Treated Prawns

3.3.1. Culture Preparation of *V. parahaemolyticus* and Prawn Infection

V. parahaemolyticus bacteria were grown on 25 ml of Luria Broth (LB) culture medium with 2% (w/v) NaCl. The pure culture was kept at 37° C shaken at 220 rpm in a shaking incubator over-night. As the next step, 50 µl of the LB culture was added (by pipetting) to 10 ml of a new LB with the same properties (2% (w/v) NaCl, shaken at 220 rpm, kept at 37° C). The sampling was done after 1, 3 and 5 hours. The density of *V. parahaemolyticus* in each sample was determined by optical density (OD) at 600 nm and then a 10 time dilution was done on each of them. 50 µl of diluted bacterial suspension was spread on a new LB agar with 2% (w/v) NaCl and was maintained at 37 °C over-night. The number of colonies appeared on each sample culture was counted considering each colony as a colony forming unit (cfu). Based on obtained information, the cfu existing in each ml of culture medium was calculated.

Prawns were divided into different groups of three and each group was infected by 100 µl of one of three diverse bacterial concentrations. 100 µl of bacterial suspension was injected into the first abdominal segment of each prawn using syringe 29G x ½" with no post-infection feeding. Prawns were monitored for 48 hours and the number of

survivors was recorded. The most minimum bacterial concentration which resulted in the highest prawn survival rate was selected to be used in experiment.

3.3.2. Prawn Infection with *V. parahaemolyticus* Time-Based Sampling

As demonstrated in Table 3.3. prawns were grouped based on different time points (each group consisted of 3 prawns). Group 1 and group 2 were injected by 100 µl of *V. parahaemolyticus* and PBS respectively. Those injected with 100 µl of PBS were maintained as negative controls. The injection was done (at 0 hour) at their first abdominal segment using a sterile syringe 29G x ½". At each time point, three prawns from infected and non-infected groups were collected to undergo dissection. Gill, hepatopancreas, pleopods, hemolymph, stomach and muscle were sampled from each prawn and were immediately dropped in liquid nitrogen to be snap-frozen. All samples were then preserved at -80 freezers prior to total RNA isolation.

Table 3.3. Group 1 (G1): "*Vibrio*" indicating the prawns injected with, *Vibrio parahaemolyticus*; Group 2 (G2): "PBS" represents prawns were injected with PBS solution as control.

Timepoints	0hpi	3hpi	6hpi	12hpi	24hpi	48hpi	Total
G1 (*Vibrio*)	3	3	3	3	3	3	24
G3 (PBS)	3	3	3	3	3	3	24

Samples were prepared in 3 duplications and the result of experiment has been represented as relative fold of each sample as mean ± standard deviation.

3.3.3. Relative Quantification of Putative CP Gene Expression in Stomach of Infected Prawns Using qPCR and Statistical Analysis:

Relative qPCR using SYBR Green Chemistry was performed to analyze the differential CP expression level in triplicates from stomach of infected prawns at different time-points i. e. 0, 3, 6, 12, 24, 48 hours after bacterial injection. The same qPCR was done to measure differential expression of CP in three replicates from PBS treated prawns at

0, 3, 6, 12, 24, 48 hours after injection of PBS (negative controls). As previously mentioned, EF-1α was used to normalize the PCR analysis and ΔCt values relevant to each time point were mean of 3 replicates with standard deviations. According to this method, ΔCt value was defined as subtracting average Ct value of housekeeping gene (EF-1α in this study) from average Ct value of putative CP gene. Differential expression of putative CP gene has been represented by comparing relative expression values of CP (mean $2^{-\Delta\Delta Ct}$) in stomach of *Vibrio* challenged prawns against those treated by PBS (negative controls) at different time points. The gene expression analysis was carried out using $2^{-\Delta\Delta Ct}$ ((Livak and Schmittgen, 2001) while ΔCt value of first time point (0 hour) was used as calibrator.

CHAPTER 4

RESULT

4.1. Identification of Putative Clotting Protein Gene in *M. rosenbergii*

4.1.1. Sequence Analysis of *M. rosenbergii* Putative CP Gene:

Clotting protein gene was identified from the *M. rosenbergii* transcriptome unigenes obtained by Illumina's Solexa sequencing technology. The CP gene sequence has been submitted to BankIT on NCBI in 20 Jan 2013. ExPASy software was used to translate the nucleotide sequence of putative CP into its deduced amino acid sequence (Gasteiger *et al*., 2003).

The nucleotides along with deduced peptide sequences from coding region of putative clotting protein gene in *M. rosenbergii* have been represented in Appendix D.

Analysis of putative CP structure revealed that the nucleotide sequence of putative CP gene in *M. rosenbergii* is 5609 base pair (bp) long with an ORF of 5139 bp. The ORF is capable of encoding a polypeptide of 1712 amino acid (aa) starts from the nucleotide 74 to 5212 with an estimated molecular mass of 189.9 k Da and a predicted pI of 5.44.

4.1.2. Prediction and Analysis of Domains in Putative CP Structure

Structural analysis of CP sequence using PROSITE suggested the presence of two domains (vitellogenin and von Willebrand factor type D domain) in putative clotting protein of *M. rosenbergii*.

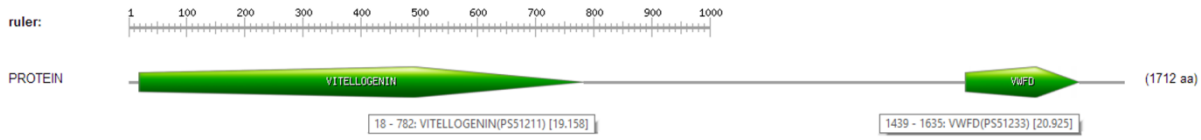


Figure 4.1. Analysis using PROSITE to identify the domains on the CP protein. The analysis shows that the positions from 81 to 782 correspond to the vitellogenin domain and positions from 1439 to 1635 correspond to the VWFD domain.

4.1.3. Identification of Signal peptide Cleavage Site for put. Clotting Protein

It has been suggested that intracellular and extracellular interactions with protein transport system resulting in protein targeting is determined by the properties of variable short peptide stretch termed as signal peptide or signal sequence which occurs in N-terminus of most of secretory proteins and is cleaved and activated by a specific enzyme called signal peptidase. Despite the presence of signal sequence in majority number of membrane-bound proteins (type I), most of multi-spanning and also type II membrane proteins lack such peptide stretch; although, their first transmembrane domain provides a biochemical signal towards their destination in secretory pathway.

Hence, in this study, we have first run the Kyte-Doolittle hydropathy plot to check for any transmembrane regions in our protein (Figure 4.2.). Then we ran Signal-P online to check “location of signal peptides cleavage sites”. The result has been shown in Figure 4.3.

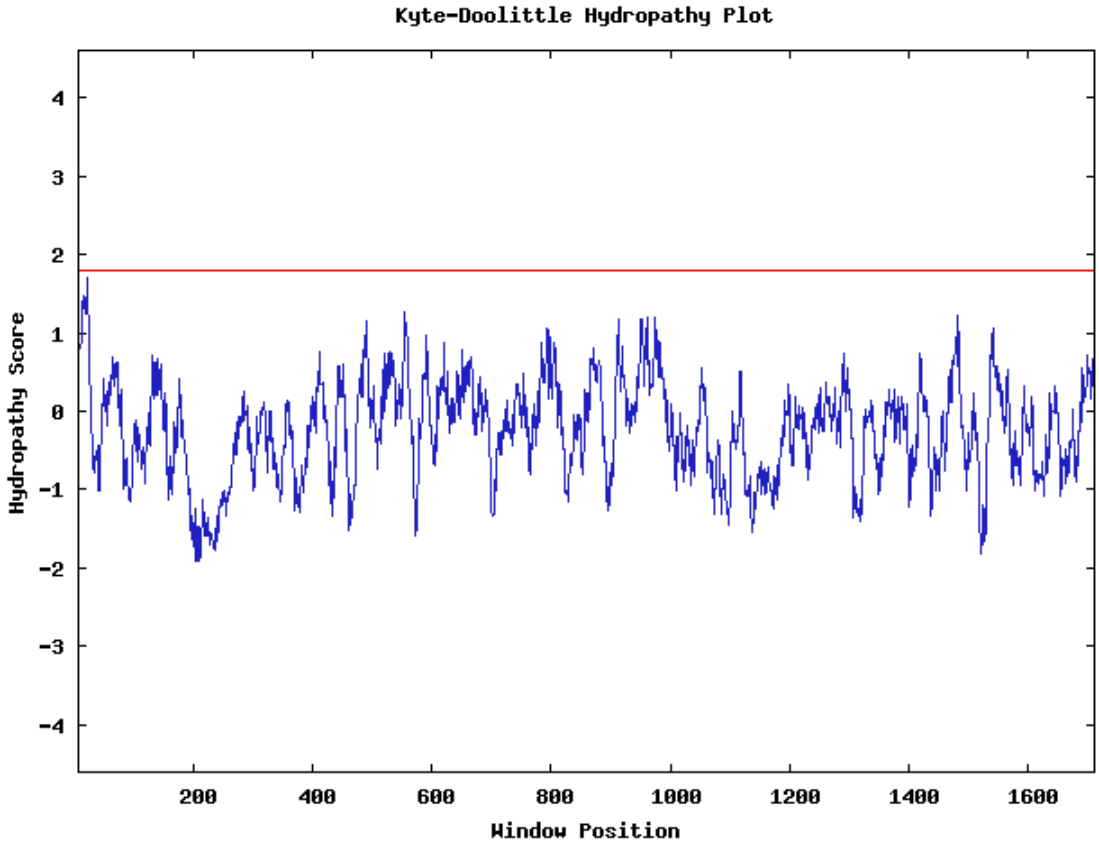
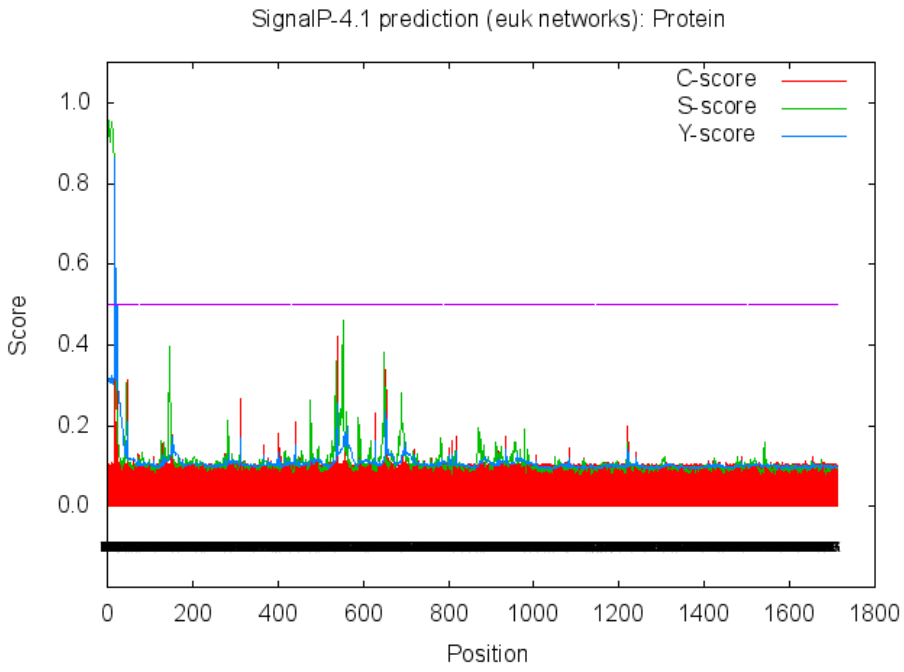
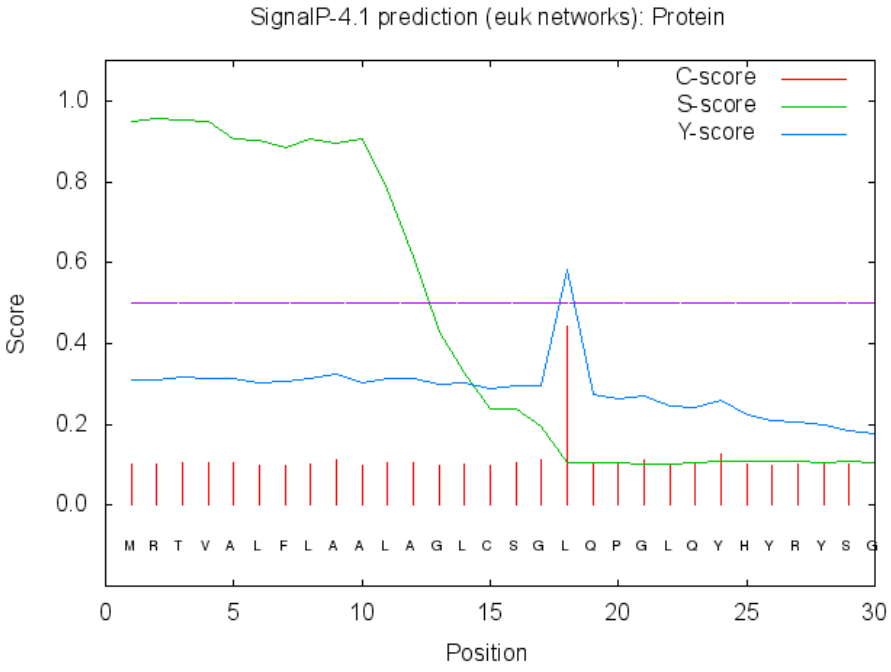


Figure 4.2. Kyte-Doolittle Hydrophathy plot.

Kyte-Doolittle Hydrophathy plot with a window size of 19 and a red line indicating a hydropathy score of 1.8. Picks which can cross the red-line represent possible transmembrane sites. As seen in the figure, our put. Clotting protein does not visibly show any transmembrane regions here.



A.



B.

Figure 4.3. Signal peptide 4.1 prediction for CP.

A. Figure representing SignalP-4.1 prediction of the whole put. clotting protein (1741a.a) and the cleavage site between positions 17 and 18 of the protein B. Figure representing the zoomed in view of the put. Clotting protein (first 30 a.a) showing the signal peptide cleavage site between the positions 17 and 18. [C=raw cleavage site, S=signal peptide score, Y=combined cleavage site score].

4.1.4. Putative Clotting Protein Alignment against Five Similar Sequences Using Uniprot

Our sequence putative CP has been used to check for any closely related sequences on NCBI-BLASTP (Altschul *et al*., 1990) and Uniprot. As shown in Appendix E, five Sequences with good percent of similarity have been selected for further alignment on Uniprot (http://www.uniprot.org/).

4.1.5. Analysis of Putative CP Protein Using The Phyre 2 Server

Figure 4.4. demonstrates the predicted three dimensional put. Clotting protein model that was generated using Phyre 2 server to find structural similarities to empirically determined proteins exist in PDB archive (Kelley and Sternberg, 2009).

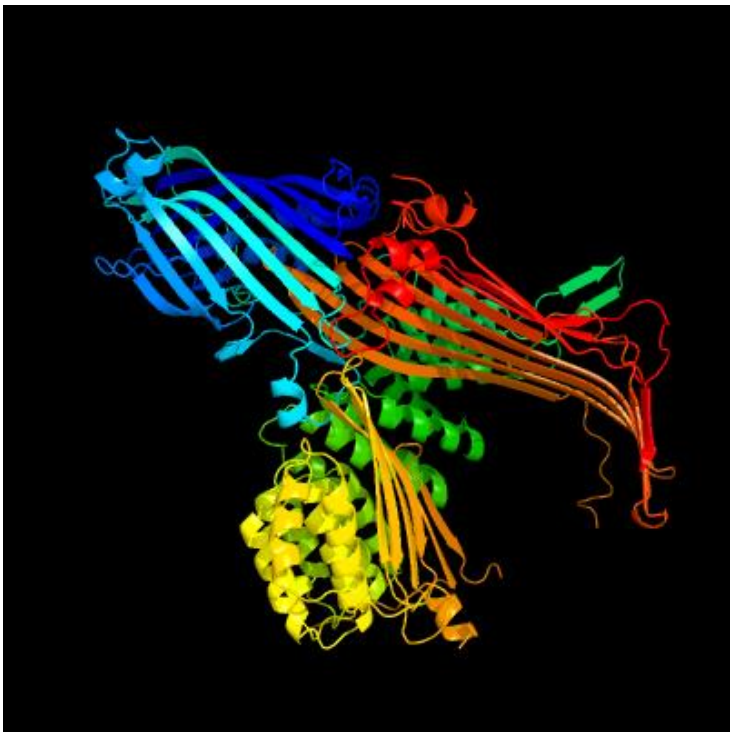


Fig. 4.4. Analysis on CP protein using the Phyre 2 server: A predicted three dimensional put. Clotting protein model with 53% coverage.

4.1.6. Construction of Phylogenetic Tree for put. Clotting protein

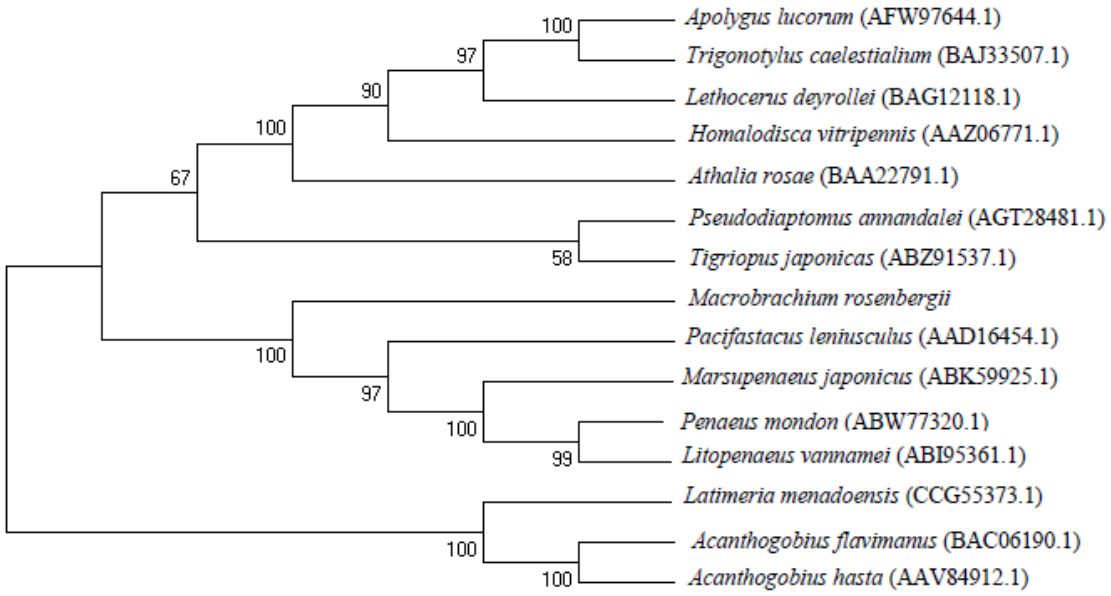


Figure 4.5. Phylogenetic tree constructed using Neighbor-Joining method at MEGA 5.05.

Neibor –Joining phylogenetic tree of Clotting protein based on the vitellogenin domain of organism group ranging from invertebrate to vertebrate constructed on the basis of Poisson model and built at a boot strap of 1000.

Phylogenetic tree was used to find closest ancestral protein to putative CP protein in *M. rosenbergii* which appeared to be tr_Q9UAR3 (*Pacifastacus leniusculus* clotting protein).

4.2. CP Primer Designing

4.2.1. Primer Designing and Optimization

A pair of real-time primer for a particular part of CP gene (a stretch from nucleotide in position 2001 to nucleotide in position 2099) was designed using Primerquest program (http://www.primerquest.com/) with 24 nucleotide length, 50% GC content, 60℃ primer TM and 99 bp product length. The primer optimization was done as previously mentioned in chapter Three. . The results were observed by AlphaImager™ Gel Imaging machine (Cell Biosciences, U.S.A) as shown in Figure. 4.6.

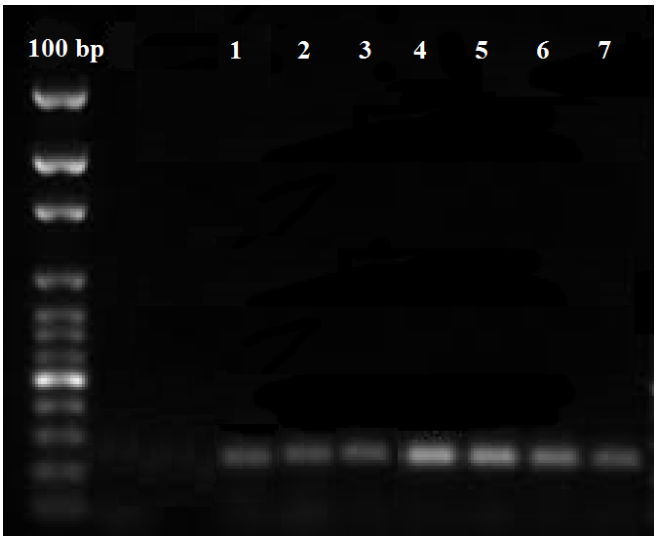


Figure 4.6. Amplification of a particular fragment of *M. rosenbergii* using new designed CP primers. Amplification reactions were prepared in total volume of 10 µl including 50 ng cDNA (extracted from gill), 1.5 mM MgCl, 1X Promega Green GoTaq® Buffer, 0.2 mM dNTPs, 0.025 U/µl Promega GoTaq® DNA Polymerase, 0.25 µM forward primer and 0.25 µM reverse primer. The primer sequences are: CP F 5' TCTGTTCTCGTTATCTGCCCGCTT 3'; CP R: 5'TGACG TGCTCCTGATGTCGTGGGATTCTT3'. The temperature profile was set as follows: 95° C for 5 minutes as an initial denaturation step, 30 cycles of 95°C for 5 minutes (denaturation), a 55 - 65°C annealing step for 1 minute, a 1 minute extension at 72°C followed by a final extension cycle at 72°C for 5 minutes. Amplification results at temperature gradients 55, 57.5, 59.7, 61.4, 63, 63.7,65 are respectively shown in lanes 1 to 7 and the marker lane contained 100bp Promega DNA Ladder (G2101).

4.2.2. PCR Clean Up and Sequencing

The amplified sequence is verified as a segment of putative CP sequence, the PCR product was subject to a clean-up using NucleoSpin® Extract II PCR clean-up kit followed by the guideline provided in Appendix B. The purified PCR product was collected and sequenced by 1st BASE *(*http://www.base-asia.com/dna_sequencing/) using BigDye® Terminator v3.1 cycle sequencing kit chemistry. The result was fully aligned against the nucleotide stretch from 2001 to 2099 using MEGA5 (Appendix C) (Tamura *et al*., 2011).

4.2.3. Optimization of Primer Concentration in Real-Time PCR

The efficiency of primer sets used in qPCR amplification is significantly important for the subsequent analysis to be statistically valid. For this validation, the primer sets need to show approximately equal performance during amplification. In order to determine the efficiency of each primer (CP and EF-1α), the calibration curve was built for three different primer concentrations (0.6 mM, 0.3 mM and 0.15 mM) based on absolute qPCR results of 5 reactions with different cDNA concentrations (cDNA concentrations on X-axis and the average of Ct value on Y-axis). The m-value was determined according to the curve slope Primer efficiencies for EF-1α (as an endogenous primer) and CP (as target) were calculated using the equation E=10 [–1/slope]-1 (Pfaffl, 2001) and the results were compared. The calibration curves of real-time PCR reactions with acceptable efficiencies result in slopes ranging from -3.10 to -3.58 (Pfaffl and Hageleit, 2001). As illustrated in figure 4.7. CP and EF-1α primers both in concentrations of 0.3 mM resulted in the best linear standard curves with acceptable slopes. These two concentrations which also show closest efficiency values were used for further experimental analysis.

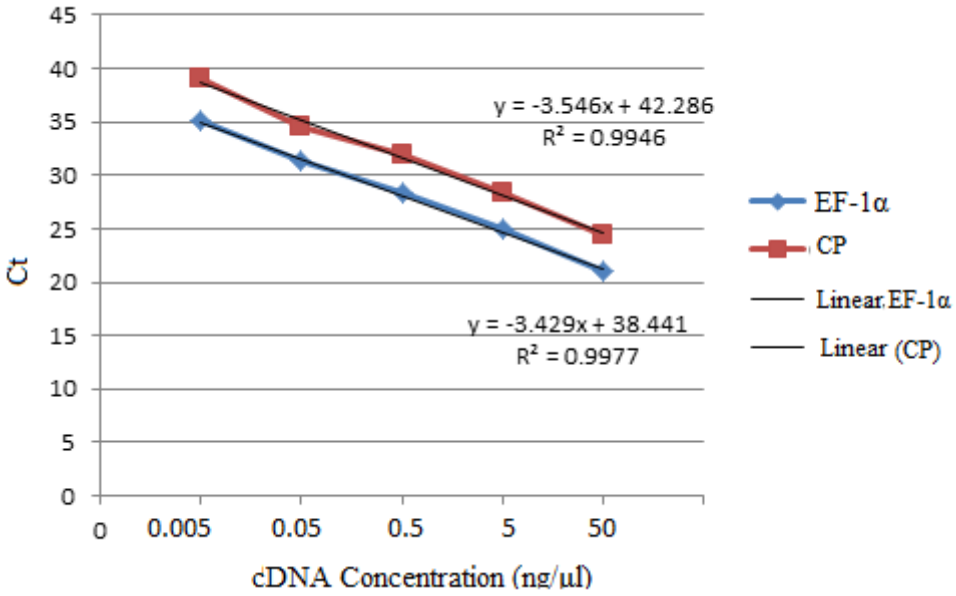


Figure 4.7. Calibration curves for CP and EF-1α at 0.3 mM concentration. cDNA concentrations are shown on X-axis and the average of Ct values are demonstrated on Y-axis. According to the formula of efficiency E=10 [–1/slope]-1, the E value for primer sets was determined as 1.82 and 1.92 for CP and EF-1α primers respectively which were among the acceptable range (1.60- 2.10) suggested by Pfaffl and Hageleit, 2001. As shown, the resulted standard curves in mentioned concentration are linear with a dispensable efficiency difference suggesting a valid performance for further experimental and statistical analysis.

4.3. Tissue expression profile using real-time PCR:

The optimized concentrations of CP and EF-1α primers (0.3 mM) were used to perform real-time PCR (relative quantification) using 50 ng/µl cDNA of six different tissues obtained from non-infected prawns. . The comparative Ct method $2^{-\Delta\Delta Ct}$ was used to determine the CP expression level in each sample tissue (Livak, 2001) and the result suggested predominant expression of putative CP gene in stomach of *M. rosenbergii.*

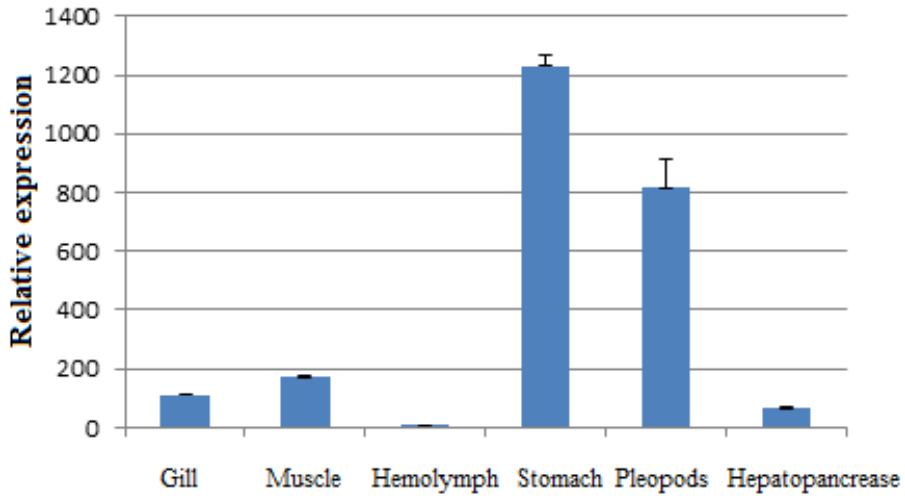


Figure 4.8. Gene expression pattern of putative CP gene by qRT-PCR. Tissue distribution of putative CP in six organs i. e. muscle, gill, stomach, pleopods, hemolymph from heart and hepatopancreas of healthy *M. rosenbergii*. Result data are demonstrated as ratio to putative CP mRNA expression in hemolymph. Stomach has shown the most expression of putative CP gene and pleopods at the second place.

4.4. Differential expression of putative CP in stomach of *vibrio* challenged and PBS treated prawns

Bacterial suspension of *Vibrio parahaemolyticus* with 1.0 X 10^5 CFU/ml was used to infect the prawns and putative CP cDNA of stomach (in different post infection time points) were subject to relative qPCR to be analyzed for differential expression. The procedures have been elaborated in chapter 3, methodology. As illustrated in figure 4.10. the putative CP mRNA expression in stomach represented a dynamic change against *Vibrio parahaemolyticus* challenge suggesting a close correlation between immune defence and clotting protein gene expression in *M. rosenbergii*.

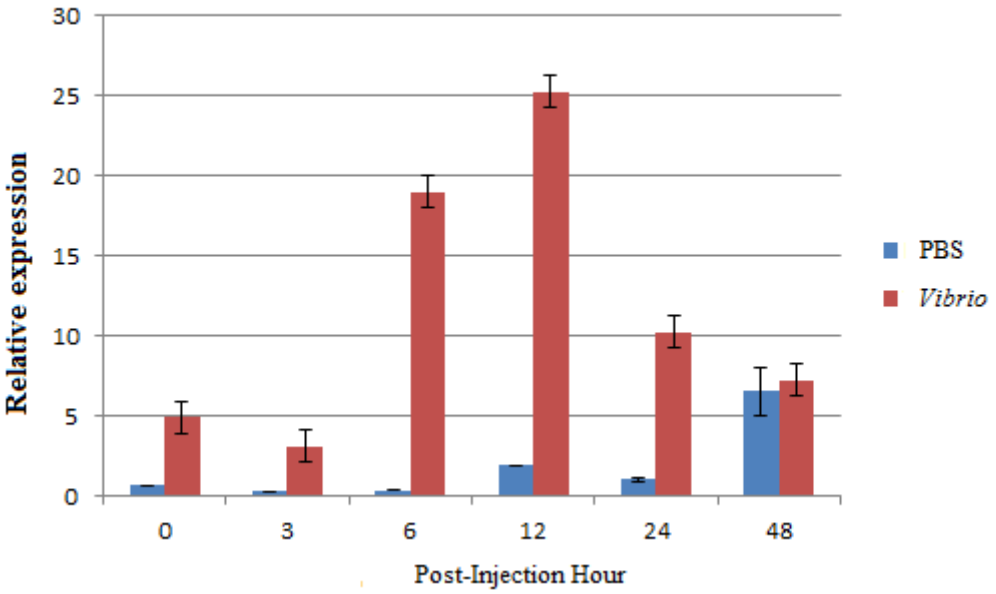


Figure 4.9. Time course of putative CP mRNA expression in stomach at 0, 3, 6, 12, 24, 48 hours post injection with *Vibrio parahaemolyticus*. Samples treated by PBS were used as negative controls. The relative quantification study at stomach showed an increasing pattern of putative clotting protein mRNA transcription until 12 hours and afterwards CP expression starts to decline.

CHAPTER 5

DISCUSSION

Shrimps constitute the great value of exported aquaculture products in recent years especially in Asia (Bhaskar *et al*., 1998). The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879) (or scampi), has been known as the most popular freshwater prawn culture in Malaysia and is playing an influential role in economy due to its wide fishery resource and indispensable commercial importance (Ng, 2007).

Reports carried out recently refer to marine *vibrios* as the major cause for bacterial disease and the major setback of aquaculture. Many studies which have been conducted on prevalence of *V. parahaemolyticus* strains in shrimps revealed 7 to 8% of fishery products contaminated by virulent strains of *V. parahaemolyticus* in tropics such as Malaysia (Sujeewa *et al*., 2009).

At a Bioinformatics point of view in this study, a putative clotting protein gene (CP gene) was sequenced from the fresh-water prawn transcriptome unigenes using Illumina's Solexa sequencing technology. Putative clotting protein gene consisted of 5609 base pairs with an open reading frame (ORF) length of 5139 bp. Putative CP consisted of 1712 amino acids with an estimated molecular mass of 189.9 k Da and predicted isoelectric point of 5.44. Closely related sequences were identified using NCBI-BLAST (Altschul *et al*., 1990) and have been used for alignment using Uniprot (http://www.uniprot.org/). Our sequence, putative CP aligned with B5KMA2 (*Penaeus monodon* clottable protein), A7YIH6 (*Penaeus japonicus* clottable protein), A8DR94 (*Litopenaeus vannamei* hemolymph clottable protein), Q9U572 (*Penaeus monodon*

hemolymph clottable protein) and Q9UAR3 (*Pacifastacus leniusculus* clotting protein). Signal peptides and domains were identified. The result of DNAstar protein analysis indicated that CP is hydrophilic and it also shows good antigenicity index. PROSITE analysis indicated the existence of vitellogenin and VWFD domains in putative CP structure in positions 81-782 and 1439-1635 respectively.

Neibor –Joining phylogenetic tree of Clotting protein based on the vitellogenin domain of organism group ranging from invertebrate to vertebrate constructed on the basis of Poisson model and built at a boot strap of 1000. Phylogenetic tree was used to find closest ancestral protein to putative CP protein in *M. rosenbergii* which appeared to be tr_Q9UAR3 (*Pacifastacus leniusculus* clotting protein).

5.1. Bacterial Infection and Global Warming:

Interestingly, the elevated rate of bacterial infection is highly linked to the most important environmental and also humanitarian crisis of 21st century called Global Warming. Atmosphere has been overloaded with carbon dioxide and resulted in significant disruption in climate with disastrous and costly consequences. Therefore, coming up with cleaner source of energy and novel ideas to reduce carbon emissions can be our biggest mission to pass on healthier earth and climate to the next generation of all living things to rely upon (Austin *et al*., 2013).

Besides changes happening in the earth climate due to global warming, oceans are affected by this hazardous phenomenon as well. Polar ice caps are dramatically melting and reducing the salinity of waters near the poles, while high weather temperature increases the rate of evaporation in areas far from poles resulting in more brininess in those regions. Under such circumstances, the oceans become more favorable place – even in high latitudes - for bacteria like *V. parahaemolyticus* which need heat and salt

contents for an optimum growth. As a consequence, some serious disease would be awaiting ocean-living creatures and human health (Belkin, 2009).

5.2. The Importance of Midgut Immunity:

Newly done studies under supervision of Donald Lightner in University of Arizona has revealed a unique and orally transmitted strain of *Vibrio parahaemolyticus* as EMS/AHPNS pathogen. China has been called the birth place of EMS since that was where EMS/NHPS got reported for the first time in year 2009. Although, the negative impact of the disease was not limited to Chinese shrimp market due to the outspread to neighbor countries such as Malaysia, Thailand and Vietnam with up to 70% mortality in farmed-shrimp and an estimated financial loss of a billion dollar per year (GOAL, 2013).

The emerged disease is very difficult to be controlled and once ingestion occurs the gastrointestinal tract gets localized by the illusive pathogen and the digestive organs like hepatopancreas get destructed and cease to function as an effect of toxin produced by *Vibrio parahaemolyticus* (Martin *et al*., 2004).

Secretion of a toxin like protein called zonula occludens toxin by ingested *Vibrio parahaemolyticus* in infected shrimp negatively affects the midgut trunk (MGT) leading to disassembly of intercellular occluding junctions. Following such destruction, detachment of MGT epithelium cells ensues. Since MGT epithelium is where the secretion of peritrophic membrane occurs, epithelium elimination might result in a restricted penetration of ingested bacteria to deeper tissues of the shrimp body besides heavy disruption in osmoregulatory system of the MGT (Verschuere *et al*., 2000).

Degranulation of the hemocytes has been seen in basal lumen of MGT during infectivity studies in the shrimp exposed to *V. parahaemolyticus* as another immune response to bacterial invasion and this is while granules of hemocytes are fraught with variety of

anti-microbial compounds, prophenoloxidase, lysosomal enzymes and peroxinectin which contribute to survival of the invaded shrimp (Martin *et al*., 2004).

Heavily infected shrimps demonstrate lethargy and weakness, incapability of the blood to clot, visible and degenerative changes in digestive glands (DG), lymphoid organs and gills. Histopathology tests conducted on shrimp immersed in water contaminated with *V. parahaemolyticus* have also shown that digestive glands undergo particular morphological changes due to bacterial invasion including hemocyte degranulation, loss of epithelium in MGT lumen and tissue necrosis (Martin and Chiu, 2003; Martin *et al*., 2004).

5.3. The Importance of Stomach in Midgut Immunity

Recently done histopathology studies showed that hepatopancreas of infected shrimp was the first and only organ negatively affected at initial phase of bacterial infection. Observation of HP malfunction before any indication of illusive agent became the impetus for further research to be conducted with the results referring to stomach of *vibrio*-positive shrimp as the possible source of the causative bacteria and the relevant toxin (Martin *et al*., 2004).

In the current study we have reported the infection of *V. parahaemolyticus* on *M. rosenbergii.* As infection persisted, the CP was isolated from hepatopancreas, pleopods, gill, muscles, stomach and hemolymph. The CP gene expression was observed in all mentioned tissues while stomach demonstrated the highest CP expression before infection. Hence, stomach was selected for further real-time studies on differential putative CP expression which were conducted in diverse time courses after infection.

Recently done studies have reported that bacterial infections in shrimp have the capability of changing the host cell gene expression. In the current study, *Vibrio*

parahaemolyticus infection significantly induced expression level of putative clotting protein in stomach of prawn and it varied in different time points after injection.

We also observed that CP mRNA transcription is highly expressed in stomach of *M. rosenbergii* at different time courses with respect to the infection caused by *Vibrio parahaemolyticus*. Result of gene expression study proved that putative clotting protein is involved in immunity action of *Macrobrachium rosenbergii* and the function against *Vibrio parahaemolyticus* infection. Similar results were reported in penaid shrimp *Sicyonia ingentis* infected by *Vibrio parahaemolyticus* and *Vibrio harveyi* (Martin *et al*., 2004).

The relative quantification study showed an increasing pattern of putative clotting protein mRNA transcription until the 12th hour. Afterwards CP expression starts to decline which seems to be an impact of loss of MGT epithelial cells. This result shows a consistency with previously done studies. Conclusively, putative clotting protein as an important unit of immune system acts against pathogens to protect the host.

5.4. *Vibrio parahaemolyticus* Stability in Shrimp Stomach:

Recent studies revealed the negative effect of acidity on viable population of organisms in stomachs of infected shrimp which also showed a 100 fold decrease in pathogenic cells during the first two hour after ingestion. While, following ingestion of more food pH of stomach increases and reduces the rate of death in invading pathogens leading to some bacterial survival during the next 1.5 to 2 hours (Vanderzant and Nickelson, 1972; Wang *et al*., 2014).

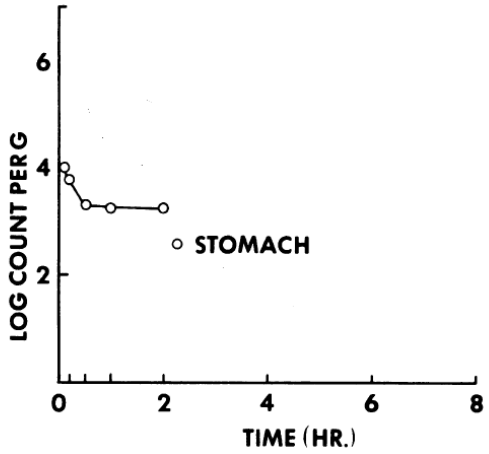


Figure 5.1. PH effect on population of *V. parahaemolyticus* inoculated into stomach of shrimp (Vanderzant and Nickelson, 1972).

5.5. Potential Risk of *Vibrio* Contaminated Shrimps Entering International Market:

According to the tests done by Lightner, it was suggested that there is a very low risk involved in EMS transmission from imported frozen shrimps to the healthy shrimp culture due to two separate reasons:
First, typically small body size of *V. parahaemolyticus* infected shrimps gives them a very low chance to enter to the international market and worldwide commerce.

Secondly, failure of attempts to induce lesions observed in frozen EMS-infected shrimps in comparison to those that were not infected indicated impossibility of this transmission.

However, further studies and investigations need to be conducted to enable hatchery and pond managements to come up with reliable diagnostic tests for *Vibrio parahaemolyticus* detection which can be considered as a long-term solution for the disease outbreak and contribute better risk evaluation in frozen shrimp importation from regions probably affected by seafood related disease.

5.6. Recommended Food Safety Strategies:

Newly published guidelines from public information sources suggested that industries involved in seafood production can ensure their food safety by applying some simple strategies; Seafood need to reach an internal temperature of 65° C for at least 1 minute. This amount can be reduced to 50° C in oysters but they need to be held in the mentioned temperature for more than 10 minutes. Foods need to be kept at either less than 5°C or more than 60° C. Reducing PH of sea-born foods to less than 4.8 is of key advice to be taken in order to meet safety objectives (Oliver and Kaper, 1997).

CONCLUSION

In the current study, the putative CP gene and its product (clotting protein) in *M. rosenbergii* were characterized using the available bioinformatics softwares. Data analysis from qRT-PCR in the tissue distribution step suggested stomach as the organ to be targeted for observation of dynamic changes in putative CP gene expression in response to *Vibrio parahaemolyticus* infection and indicated the crucial role of stomach in gut immunity.

The statistical analysis of relative quantification RT-PCR revealed differential expressions of the putative CP gene at different intervals after bacterial infection implying the close association of CP with the prawn immunological response.

In several studies previously done, midgut and gill have been referred to as the main route for pathogen uptake in crustaceans. For instance, the *Vibrio parahaemolyticus* infected shrimps demonstrated inflammation and degenerative changes in gut along with hepatopancreas (Martin *et al*., 2004). Having done experimental tests regarding to crustacean gut immunity, some important changes were observed in gene expression following the occurrence of infection (Soonthornchai *et al*., 2010). As shown in the current study, putative CP gene undergoes changes in expression level in stomach selected as the experimental target tissue.

Crustaceans' gut being the primary route for entrance of infectious pathogens has been suggested for oral administration of various types of prophylactics as well. Interestingly, with information about gut immunity novel nutrition regulatory systems, beneficial disease control strategies have been developed. Hence, better understanding of gut

immunology without doubt will help improve the idea of probiotic application with the aim of enhancing immune protection and health in farmed crustacean species (Verschuere *et al*., 2000).

Directions for Further Researches

The phenomenon of global warming and rapidly growing list of infectious diseases among decapoda have significantly intensified the need for new prophylactic treatment strategies with explicit understanding of bio-molecular cornerstones. The aim should be set to block localized or systemic infection through precisely calculated interventions in the crustacean immune system.

The complexity of the invertebrate immune system and also the disease cycle itself have made direct manipulation far-fetched in the fisheries sector. Furthermore, lack of structural and functional information on the diverse antimicrobial peptides involved in immune reactivity and their expression changes in different time courses of microbial invasion clearly show the great need for more detailed research.

Moreover, over-attention to hemocytes and hemolymph -which were believed to be the main part of immune activity- have kept the available information of gut immunity very limited in crustacean species. Hence, the augmentation of the immune defence mechanism in the gut area will definitely benefit strategies made for disease mitigation (Aleivar-Warren, 2001; Lightner, 2005).

Such novel approaches can be applied to warrant the maintenance of sustainable fisheries in depth of a ‘future ocean’ that is believed to become dramatically impacted by climate change and its consequences such as modified infectious pathogens (Harvell *et al*., 2002; Ford and Smolowitz, 2007).

APPENDIX A

Total RNA Extraction

1 ml of Trizol was pipetted into 1.5 ml micro-centrifuge tube and incubated at room temperature. Sample tissues were frozen by liquid nitrogen and then ground using autoclaved mortar and pestle. A small amount of tissue powder was transferred to the tubes containing Trizol, was shaken gently and left at room temperature for about 5 minutes. Then 200 µl of chloroform was added to each micro-centrifuge tube mixed vigorously for about 15 seconds. After 10 minutes incubation at 25 °C, tubes were centrifuged at 12000 rpm at 4°C for 15 minutes. Then aqueous layer was pipetted into a new 1.5 ml centrifuge tube and 500 µl of isopropanol (ice-cold) was added to every tube and then kept at room temperature for another 10 minutes. Next centrifugation was performed at 12000 rpm for 8 minutes at 4°C and supernatant was thrown away. About 1ml of ice-cold 75% ethanol was added to centrifuge tubes containing pellets. The last centrifugation was done at 7500 rpm at 4 °C for 5 minutes and supernatant were decanted. Pellets remained at tubes were left under laminar hood for 5 minutes to air-dry at room temperature. In the final step, about 40 µl of nuclease free water was added to the tubes to re-suspend pellets. Tubes were preserved at a freezer with maintained temperature of -80 for later use. Spectrophotometric analysis was done using nanodrop (light adsorption at Abs 260/280) to measure the quantity and quality of extracted RNA assuming a good quality at ratios of 1.6 to 2.1.

APPENDIX B

PCR clean-up

After running PCR product on a gel electrophoresis, the lane including desired fragment of DNA was carefully cut out under UV light. Extracted gel purification was done using NucleoSpin® Extract II PCR clean-up kit as directed by provided protocol:

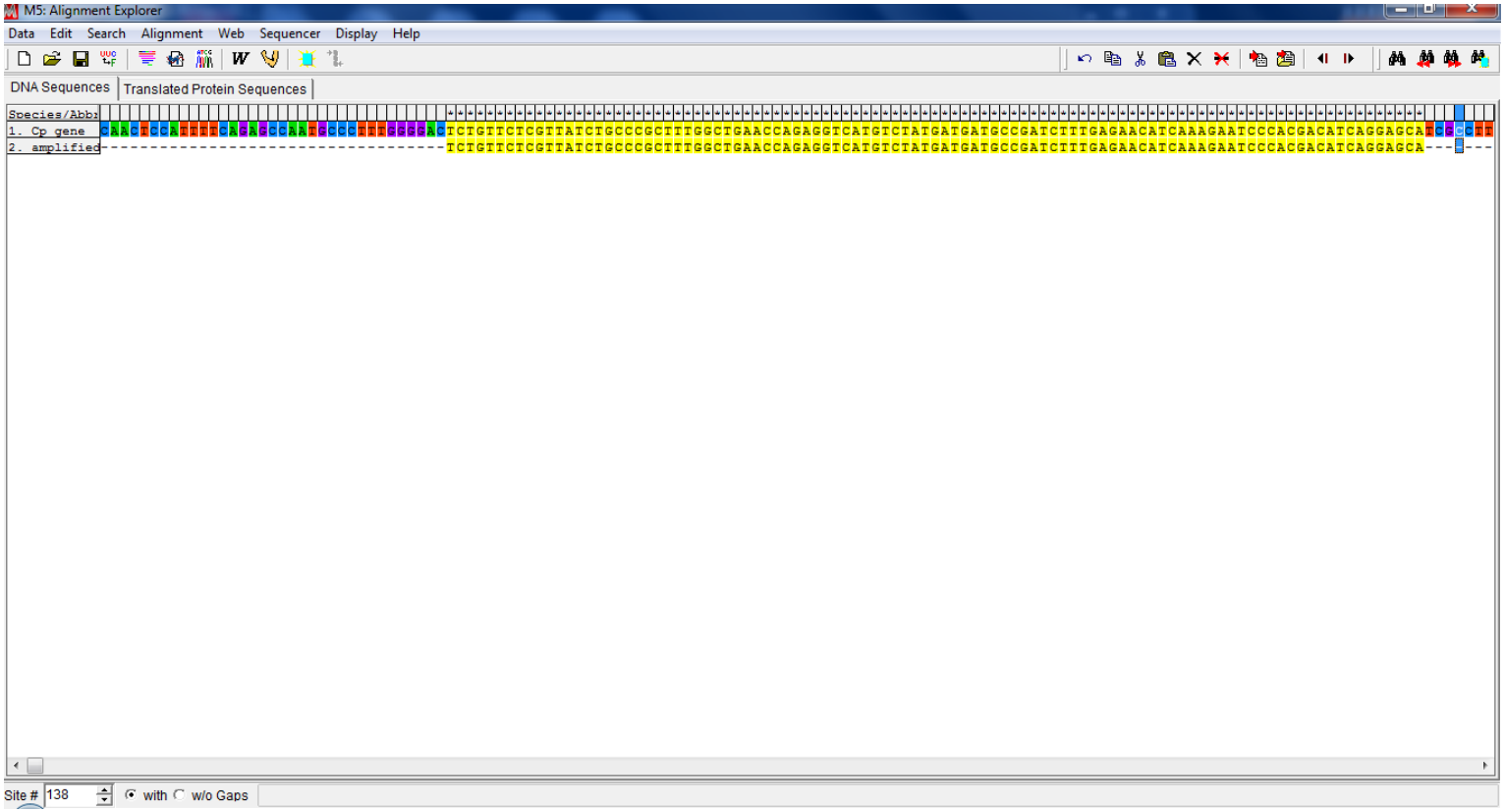
30 µl of sample was mixed with 60 µl of Buffer NT1 in a PCR clean-up column and it was placed into a collection tube followed by performing a 30 s centrifuge at 11,000 x *g*. The flow-through was thrown away and the centrifuge step was repeated.

In washing step, 700 µl of Buffer NT3 (which was already mixed with ethanol 97%) was added to the column and another 30 s centrifuge was run at 11,000 x *g* and the flow-through was discarded.

In order to dry the membrane we continued the procedure by performing another centrifuge for 60 s at 11,000 x *g* which also helped clean off contamination with residual Buffer NT3.

After all done, the column was placed into a new microcentrifuge tube and Buffer NE (20 µl) was added gently on the membrane. The tube was kept at 25°C for about 5 min and the last centrifuge was done at 11,000 x *g* for 60 s.

APPENDIX C


M5: Alignment Explorer
Data Edit Search Alignment Web Sequencer Display Help
DNA Sequences
Translated Protein Sequences
Species/Abbr
1. Cp gene
2. amplified
Site # 138
with
w/o Gaps

APPENDIX D

The nucleotide sequence of putative CP gene and its derived amino acid chain. Numbering has been done from 5' end of nucleic acid sequence and each single-letter underneath triplet codon represents its corresponding amino acid abbreviation. AUG and TAA are the start and stop codons respectively and has been bolded.

```
74 atgagaacggtggctcttttcctggcggctctggccggtttgtgt
       M  R  T  V  A  L  F  L  A  A  L  A  G  L  C
   119 tccgggctgcaaccgggcctgcaatatcactaccggtacagcgga
       S  G  L  Q  P  G  L  Q  Y  H  Y  R  Y  S  G
   164 cggctggcgaccggtatcatggaagccagacatcaggtcgcgggc
       R  L  A  T  G  I  M  E  A  R  H  Q  V  A  G
   209 gctggaatacaagcggacgtcgaggtccaggtgattgaagactac
       A  G  I  Q  A  D  V  E  V  Q  V  I  E  D  Y
   254 acagttattttccagttcagtcatgtccaagtaggcgacatccac
       T  V  I  F  Q  F  S  H  V  Q  V  G  D  I  H
   299 gatgaactgcactgtgacaggagggcccctctgcccatagagtac
       D  E  L  H  C  D  R  R  A  P  L  P  I  E  Y
   344 catcgactggaaaatggggtggctttgctggaaaagcctttcaag
       H  R  L  E  N  G  V  A  L  L  E  K  P  F  K
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       V  K  I  D  Q  N  K  K  P  F  F  F  E  F  L
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1109 aaggcggttcgactcgtcgacgaaccgctggccatcgactacgaa
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     P  H  H  C  S  S  W  R  Y  E  I  E  H  S  V
1199 gccgatcaccacgctgcaggaacgcagcctagccttgaacagatc
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     K  G  *
```
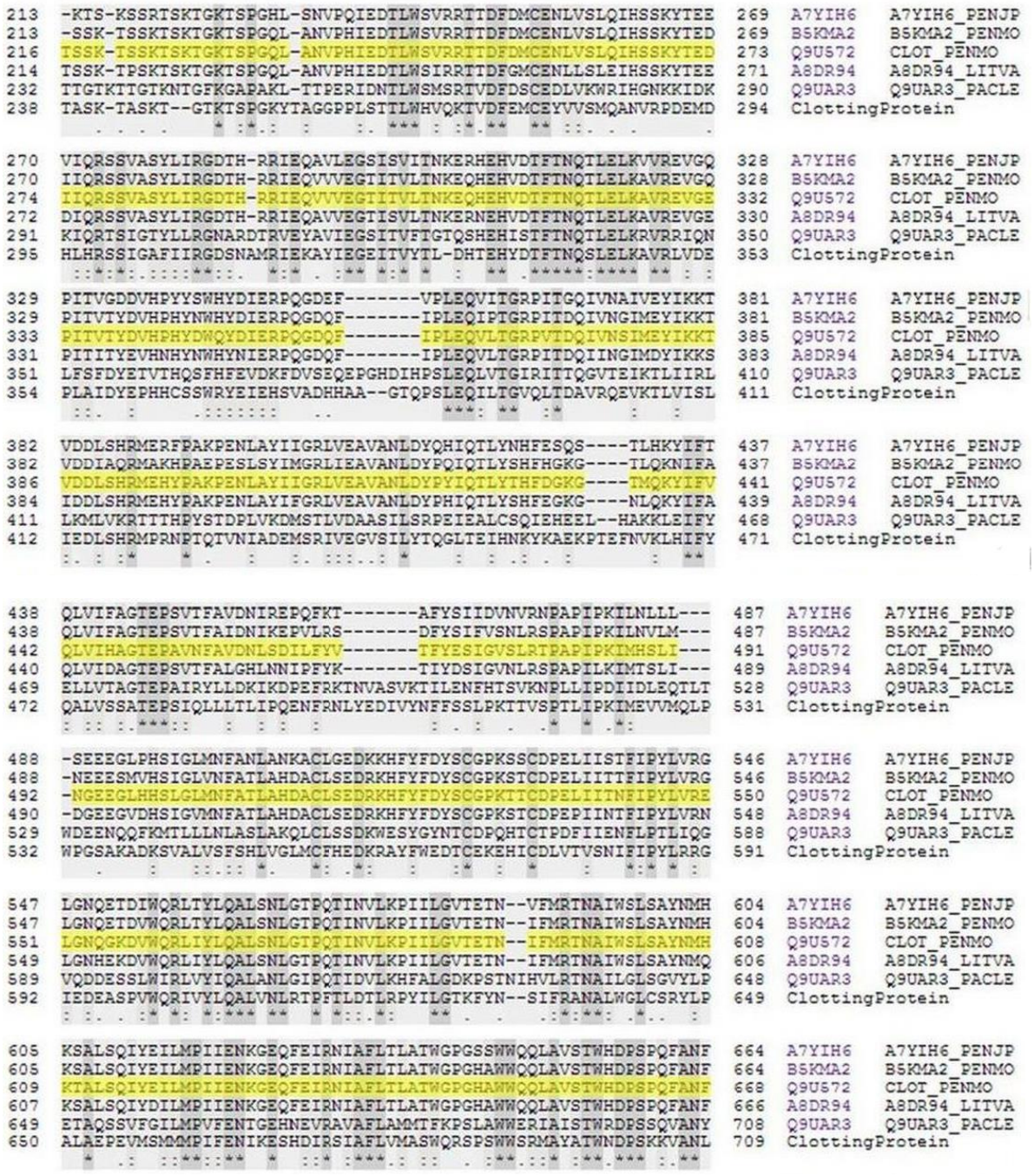

APPENDIX E

Multiple sequence alignments of putative clotting protein compared to five other homologous amino acid sequences using Uniprot. One protein sequence from *Penaeus japonicas* (A7YIH6), 2 from *Penaeus monodon* (B5KMA2 and Q9U572), 1 from *Litopenaeus vannamei* (A8DR94) and 1 from *Pacifastacus leniusculus* (Q9UAR3). The Q9UAR3 sequence highlighted in BLUE represents the signal peptide while the regions highlighted in yellow represent the domains of the protein and the correspondence to its similar sequences.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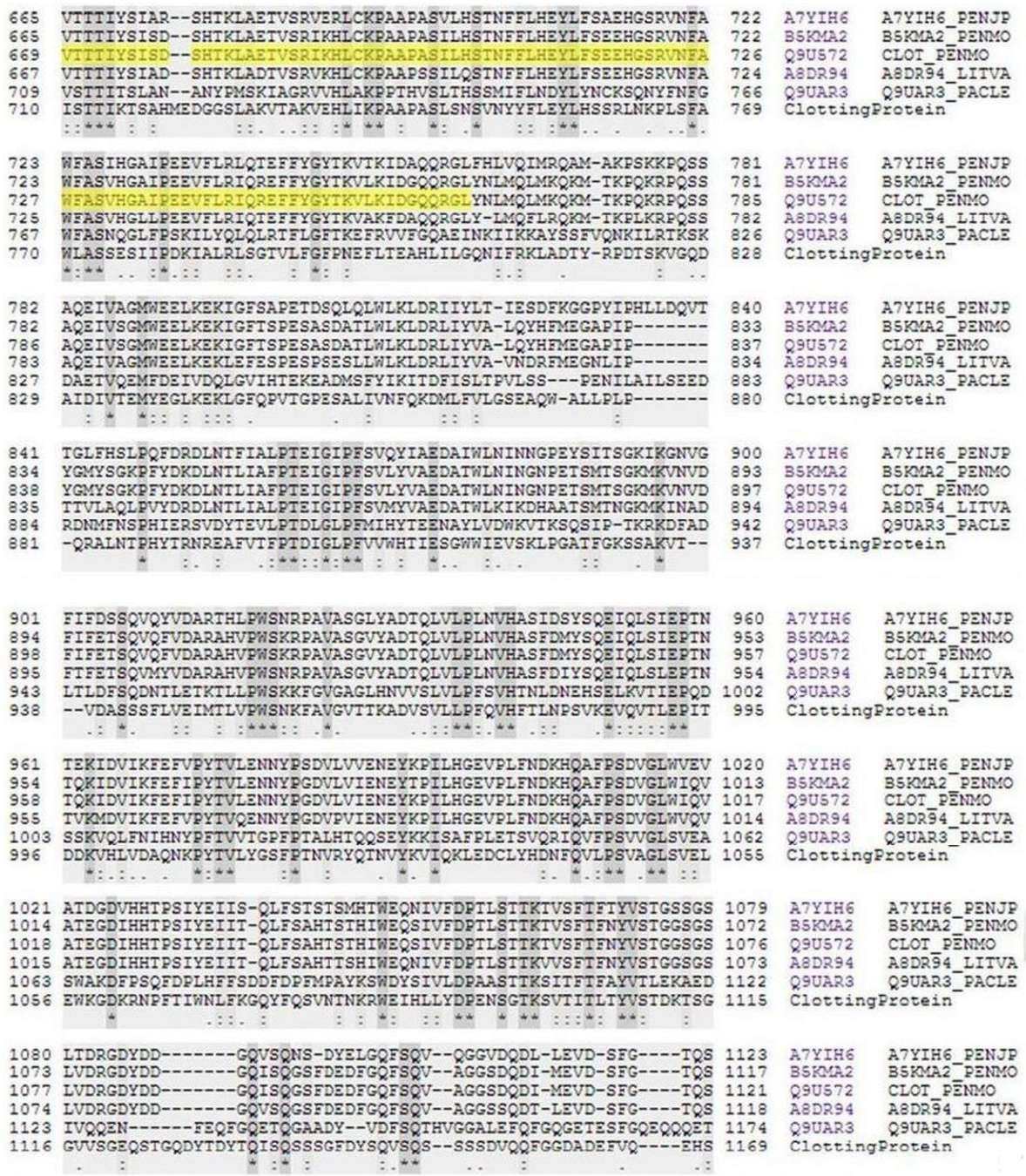
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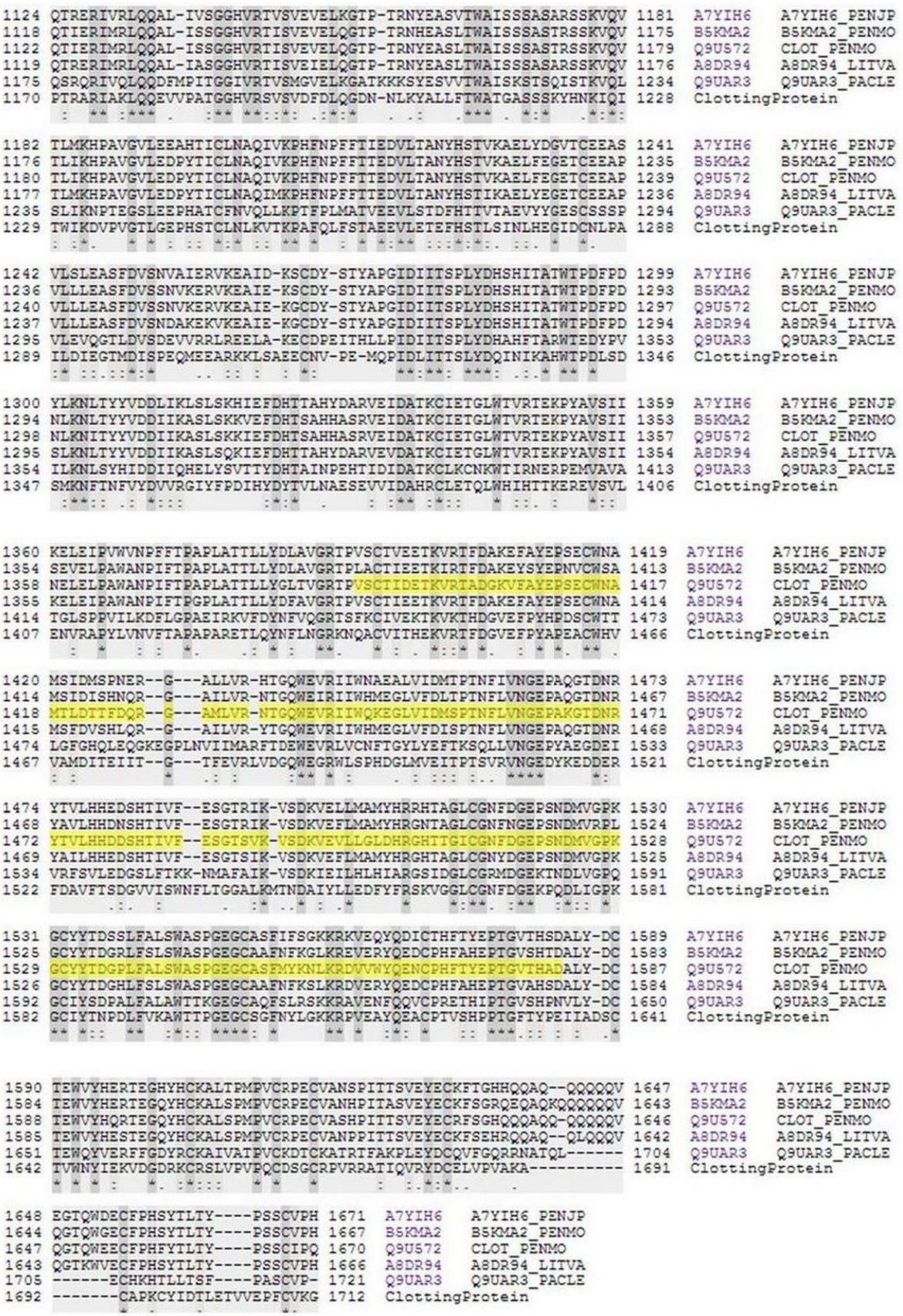
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1474 LGFGHQLEQGKEGPLNVIIMARFTDEWEVRLVCNFTGYLYEFTKSQLLVNGEPYAEGDEI 1533 Q9UAR3 Q9UAR3_PACLE
1467 VAMDITEIIT--G---TFEVRLVDGQWEGRWLSPHDGLMVEITPTSVRVNGEDYKEDDER 1521 ClottingProtein
1474 YTVLHHEDSHTIVF--ESGTRIK-VSDKVELLMAMYHRRHTAGLCGNFDGEPSNDMVGPK 1530 A7YIH6 A7YIH6_PENJP
1468 YAVLHHDNSHTIVF--ESGTRIK-VSDKVEFLMAMYHRGNTAGLCGNFNGEPSNDMVRPL 1524 B5KMA2 B5KMA2_PENMO
1472 YTVLHHDDSHTIVF--ESGTSVK-VSDKVEVLLGLDHRGHTTGICGNFDGEPSNDMVGPK 1528 Q9U572 CLOT_PENMO
1469 YAILHHEDSHTIVF--ESGTSIK-VSDKVEFLMAMYHRGHTAGLCGNYDGEPSNDMVGPK 1525 A8DR94 A8DR94_LITVA
1534 VRFSVLEDGSLFTKK-NMAFAIK-VSDKIEILHLHIARGSIDGLCGRMDGEKTNDLVGPQ 1591 Q9UAR3 Q9UAR3_PACLE
1522 FDAVFTSDGVVISWNFLTGGALKMTNDAIYLLEDFYFRSKVGGLCGNFDGEKPQDLIGPK 1581 ClottingProtein
1531 GCYYTDSSLFALSWASPGEGCASFIFSGKKRKVEQYQDICTHFTYEPTGVTHSDALY-DC 1589 A7YIH6 A7YIH6_PENJP
1525 GCYYTDGRLFSLSWASPGEGCAAFNFKGLKREVERYQEDCPHFAHEPTGVSHTDALY-DC 1583 B5KMA2 B5KMA2_PENMO
1529 GCYYTDGPLFALSWASPGEGCASFMYKNLKRDVVWYQENCPHFTYEPTGVTHADALY-DC 1587 Q9U572 CLOT_PENMO
1526 GCYYTDGHLFSLSWASPGEGCAAFNFKSLKRDVERYQEDCPHFAHEPTGVAHSDALY-DC 1584 A8DR94 A8DR94_LITVA
1592 GCIYSDPALFALAWTTKGEGCAQFSLRSKKRAVENFQQVCPRETHIPTGVSHPNVLY-DC 1650 Q9UAR3 Q9UAR3_PACLE
1582 GCIYTNPDLFVKAWTTPGEGCSGFNYLGKKRPVEAYQEACPTVSHPPTGFTYPEIIADSC 1641 ClottingProtein
1590 TEWVYHERTEGHYHCKALTPMPVCRPECVANSPITTSVEYECKFTGHHQQAQ--QQQQQV 1647 A7YIH6 A7YIH6_PENJP
1584 TEWVYHERTEGQYHCKALSPMPVCRPECVANHPITASVEYECKFSGRQEQAQKQQQQQQV 1643 B5KMA2 B5KMA2_PENMO
1588 TEWVYHQRTEGQYHCKALSPMPVCRPECVASHPITTSVEYECRFSGHQQQAQQ-QQQQQV 1646 Q9U572 CLOT_PENMO
1585 TEWVYHESTEGQYHCKALSPMPVCRPECVANPPITTSVEYECKFSEHRQQAQ--QLQQQV 1642 A8DR94 A8DR94_LITVA
1651 TEWQYVERFFGDYRCKAIVATPVCKDTCKATRTFAKPLEYDCQVFGQRRNATQL------ 1704 Q9UAR3 Q9UAR3_PACLE
1642 TVWNYIEKVDGDRKCRSLVPVPQCDSGCRPVRRATIQVRYDCELVPVAKA---------- 1691 ClottingProtein
1648 EGTQWDECFPHSYTLTY----PSSCVPH 1671 A7YIH6 A7YIH6_PENJP
1644 QGTQWGECFPHSYTLTY----PSSCVPH 1667 B5KMA2 B5KMA2_PENMO
1647 QGTQWEECFPHFYTLTY----PSSCIPQ 1670 Q9U572 CLOT_PENMO
1643 QGTKWVECFPHSYTLTY----PSSCVPH 1666 A8DR94 A8DR94_LITVA
1705 ------ECHKHTLLTSF----PASCVP- 1721 Q9UAR3 Q9UAR3_PACLE
1692 -------CAPKCYIDTLETVVEPFCVKG 1712 ClottingProtein

APPENDIX F

Document Name: cp ef1a 2nd Oct res
Plate Type: Absolute Quantification
User: Administrator

Document Information

Operator: Administrator
Run Date: Wednesday October 03 2012 14:47:20
Last Modified:
Wednesday October 03 2012 17:02:19
Instrument Type: Applied Biosystems 7500 Real-Time PCR System

Comments:
SDS v1.3.1

Thermal Cycler Profile

Stage	Repetitions	Temperature	Time	Ramp Rate	Auto Increment
1	1	50.0 °C	2:00	100	
2	1	95.0 °C	10:00	100	
3	40	95.0 °C	0:15	100	

		60.0 °C	1:00	100
4 (Dissociation)	1	95.0 °C	0:15	Auto
		60.0 °C	1:00	Auto
		95.0 °C	0:15	Auto

Standard 7500 Mode
Data Collection : Stage 3 Step 1
PCR Volume: 15 µL

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	S.D.Qty	Filtered	Tm
A1	cDNA 50ng	CP 0.3	Standard	24.75						77.5
A2	cDNA 50ng	CP 0.3	Standard	24.41						77.9
A3	cDNA 50ng	CP 0.3	Standard	24.1						77.5
A4	cDNA 5ng	CP 0.3	Standard	27.89						77.9
A5	cDNA 5ng	CP 0.3	Standard	28.3						77.9
A6	cDNA 5ng	CP 0.3	Standard	28.92						77.9
A7	cDNA 0.5ng	CP 0.3	Standard	31.47	3.471					
A8	cDNA 0.5ng	CP 0.3	Standard	31.7	3.471					
A9	cDNA 0.5ng	CP 0.3	Standard	32.38	3.471					
A10	cDNA 0.05ng	CP 0.3	Standard	34.53	3.099					78.3
A11	cDNA 0.05ng	CP 0.3	Standard	34.96	3.099					78.3
A12	cDNA 0.05ng	CP 0.3	Standard	34.1	3.099					78.3
B1	cDNA 50ng	EF-1α 0.3	Standard	21.5	7.964					78.6

B2	cDNA 50ng	EF-1α 0.3	Standard	21.14	7.964	78.6
B3	cDNA 50ng	EF-1α 0.3	Standard	20.45		78.6
B4	cDNA 5ng	EF-1α 0.3	Standard	25.32	5.281	79
B5	cDNA 5ng	EF-1α 0.3	Standard	25.02	5.281	79
B6	cDNA 5ng	EF-1α 0.3	Standard	24.72	5.281	79
B7	cDNA 0.5ng	EF-1α 0.3	Standard	28.53	4.806	79
B8	cDNA 0.5ng	EF-1α 0.3	Standard	28.22		79
B9	cDNA 0.5ng	EF-1α 0.3	Standard	28.36	4.806	79
B10	cDNA0.05ng	EF-1α 0.3	Standard	31	0.248	79
B11	cDNA0.05ng	EF-1α 0.3	Standard	31.48	0.248	79
B12	cDNA0.05ng	EF-1α 0.3	Standard	31.51	0.248	79
C1	cDNA0.005ng	EF-1α 0.3	Standard	35.88	4.806	79.4
C2	cDNA0.005ng	EF-1α 0.3	Standard	34.96	4.806	79.4
C3	cDNA0.005ng	EF-1α 0.3	Standard	34.22	4.806	79.4
C4	cDNA0.005ng	CP 0.3	Standard	39.18	3.099	78.3
C5	cDNA0.005ng	CP 0.3	Standard	39.44	3.099	
C6	cDNA0.005ng	CP 0.3	Standard	40.48		78.3
C1			NTC	Undetermined		73.8
C2			NTC	Undetermined		73.8
C3			NTC	Undetermined		73.8

APPENDIX G

Real-Time PCR

Differential Expression of putative CP in Stomach of *M. rosenbergii*

	Vibrio 0	*Vibrio* 3	*Vibrio* 6	*Vibrio* 12	*Vibrio* 24	*Vibrio* 48
Ct (CP)	27.46	28.868	29.026	27.861	28.965	24.828
Ct (CP)	27.288	28.904	29.202	28.193	28.873	24.927
Ct (CP)	27.618	28.655	29.043	27.858	29.705	24.469
Average	27.4553	28.809	29.0903	27.9707	29.181	24.7413
Ct (ef1a)	22.797	23.368	26.446	25.651	25.342	20.95
Ct (ef1a)	22.834	23.563	26.243	25.525	25.43	20.286
Ct (ef1a)	22.703	23.424	26.379	25.755	25.853	20.53
	4.65833	5.441	2.64433	2.31967	3.839	3.79133
	4.62133	5.246	2.84733	2.44567	3.751	4.45533
	4.75233	5.385	2.71133	2.21567	3.328	4.21133
	14.032	16.072	8.203	6.981	10.918	12.458
	6.981	6.981	6.981	6.981	6.981	6.981
	2.32267	1.54	4.33667	4.66133	3.142	3.18967
	2.35967	1.735	4.13367	4.53533	3.23	2.52567
	2.22867	1.596	4.26967	4.76533	3.653	2.76967
	5.00256	2.90795	20.2054	25.3047	8.82747	9.124
	5.13252	3.32879	17.5533	23.1884	9.38268	5.75839
	4.68701	3.02304	19.2885	27.1962	12.5795	6.8195
Ave	4.94069	3.08659	19.0157	25.2298	10.2632	7.23397
Std	0.18707	0.17759	1.09977	1.63702	1.65346	1.40491

Real-Time PCR

Differential Expression of putative CP in Stomach of *M. rosenbergii*

	PBS0	PBS 3	PBS6	PBS12	PBS24	PBS48
Ct (CP)	29.683	27.995	28.546	29.3	36.555	28.725
Ct (CP)	29.066	28.144	28.68	29.513	36.937	28.059
Ct (CP)	29.043	27.021	28.52	29.755	36.657	28.985
Average	29.264	27.72	28.582	29.523	36.716	28.59
Ct (CP)	24.72	21.807	23.088	26.453	32.689	27.371
Ct (CP)	24.594	21.743	23.264	26.486	33.012	27.62
Ct (CP)	24.629	21.919	23.277	26.475	32.524	26.781
	4.544	5.913	5.494	3.0697	4.0273	1.2187
	4.67	5.977	5.318	3.0367	3.7043	0.9697
	4.635	5.801	5.305	3.0477	4.1923	1.8087
	13.849	17.691	16.117	9.154	11.924	3.997
	3.997	3.997	3.997	3.997	3.997	3.997
	-0.547	-1.916	-1.497	0.9273	-0.03	2.7783
	-0.673	-1.98	-1.321	0.9603	0.2927	3.0273
	-0.638	-1.804	-1.308	0.9493	-0.195	2.1883
	0.6844	0.265	0.3543	1.9018	0.9792	6.8606
	0.6272	0.2535	0.4003	1.9458	1.2249	8.153
	0.6426	0.2864	0.4039	1.931	0.8734	4.5578
	0.6514	0.2683	0.3861	1.9262	1.0258	6.5238
	0.0242	0.0136	0.0226	0.0183	0.1473	1.4869

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